Department Virology

Subject Alphavirus Replication II

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Address MEID

Lab Notebook

National Brand 3576

43-648

Computation Notebook

Dennison Stationery Products Co., Framingham, MA 01701

Issued 8-23-93

75 Sheets
11¾" x 9¼"
4x4 Quad.

0 73333 43648 8

EXHIBIT D

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Publish Report ... 2760

22.20 - R Cuttle ...
- Dig ... 20 yds Apx
- Pump
- Line
- Tools ...

All ... last Fish Apx 1 Sq ft
Dig Trench 22.20
Find ... 70° Ft ... Apx
5267 2.68

Patrick Ford for ... 6 Cal Magnet
... with Ch' ... Lg ...
... Lg ... 6 ...
Shut
- Blind ... Knoll
- 456.75'
- 25 See page 57

BEST AVAILABLE COPY

1 → Go to p. 131

Preparation of DNA fragment for injection 5/23/93

Plasmid pTG1 - Sol #5 - 10 Frozen stock - 70 Sol #10

Digested with EcoRI and Sal I

- 3 bands fragment purified by electroelution - Sol #3
- Lyophilized to 5 ul - re-dissolved in 1 ml injection buffer Sol #5 - no dilution
- Final Sol #6 - 10.3 ng/ml

Wrong Vector

Second preparation of fragment in 20 ul H2O Discarded

Digested with EcoRI

Vector Control 2 ul 10x buffer + 10 ul H2O + 8 ul DNA

DNA 2 ul 10x buffer + 5 ul H2O + 13 ul DNA

Rm T, T = 2:30

Check in microinjection log - Sol #1 - 1 ml

Transfer to replicate by digestion of appropriate plasmid with EcoRI - Sol #1

Bill Ahn 1

April injection to remove the first pronuclear which removed the hypothesized inviable phase as Nancy's map of the adenovirus suite.

Cleaned the Gene by Blunt and cloning into the filled in EcoRI site.

5-15

15ng pd220

digested with 20 units ClaI in high salt buffer

1 hour.

Phosphatase treat = 54 ng/ul = Sol #70 - 20C

Discarded 6-2-93

Blunting fragment

2ul 10x high salt 95 ul

1ul 5mM dNTP 1 ul Klenow 37C 20' → Sol #65 15'

Sol #46

Ligation of PstI 4h 22d 2:20 -5c

	Vector	Insert	10x	H_2O	Lig
Vector	1	0	3	24	2
PstI	1	5	3	19	2
DSC			3		

Digest Vector was not properly purified

1) Digest with ClaI - 5µl 10x High, 22µl H_2O, 20µl
2) Phenol Repair and Ethanol ppt, re-dissolve in 10µl
3) Run Column
4) Run Column
5) Gel Extract 90µl, 5µl 10x, 45µl Buf, 3µl, 15'
6) Run
7) Nan

$OD_{260} = 0.75 = 37.5 ng/µl$

Ligation

	Vector	Insert	10x	H_2O	Lig
Vector	1	0	3	24	2
PstI	1	5	3	19	2
	✗	✓	✓	✗	✗

RmT - 0/3 9:30p.m -

Hundreds of colonies on both Ampicillin + Kan plates

Picked 10 for minis

Digested with KbaI - 5µl

→ ~1.3Kb fragment

6.10.98

Results indicate significant amount of digested vector in insert preparation

Redo using PstI digested vector

Add glass milk prep
5µl 10x High
5µl H_2O
1.5µl KI
1.5µl SacI 37C 5'
1.5µl PstI

Where is repair protocol initialed

to P38

Put [illegible] to 40

[illegible]

Repair wire tester.

① Disconnect J2-20 — Clock to PD. 6/48
High-Set Button — Sig — Sig — Sig — Sig — Sig

② Wires for Complete & Disconnect

③ Remove Servo — 2.0 Ω
Sec Servo
Sec 20
Sec to Servo
To Sec
Short
Short Solder

④ Phase Shifter Test
Add 4µf 400 V/40 V01 Sec Sec 1/4 to 1/8
Sec Sec Cap
Sec 20
Sec to Servo
To Sec
Short
Short
Short

⑤ Power Supply Electrolytic

⑥ Lights

⑦ TV Repair
23.044
Sec Sec TV
Video
Sec TV
39° Sec Sec
Sec Sec Video
Sec Sec
Sec Sec
Sec TV — 20 min
Sec Sec
Sec Sec
Age - Electrolytic
Short 25
Swapped in 40µf 40 Volt Slot 58. Discard.
62-93

Ligation

	Vector	Ins	10x	H_2O	Lig
RVF	2	5	3	18	2
VECTOR	2	0	3	23	2
	X	X	X	—	

5-27-93

Innt HB101

- Picked 12 colonies for minipreps
- Digested with Nhe I
- Vector is predicted to release 1322bp 816 bp as shown in Lane 2
None of the 12 are as expected - additional Nhe I fragments missing except for the ~650bp fragment.

Several questions remain - Why is the 650bp frag there but not the 816

What are the plasmids in the control plate

5-30

- Pick 3 colonies of control inserts prep and B from RVF plate
- Miniprep
- Include digests of RVF → Nhe I
- Vector - Replicon
- R/H3 digests also

Predicted Nhe I frags

Replicon 1322, 816

Replicon-RVF + 1322, 816, 946, 651

− ~~537~~ 1322, ~~650~~ 816, 2401, 651 - Vector

All 6 picks show same fragment. discard vector and redo

to page 12

Helper Restriction cont'd.

Repeat restriction digest of 272-3 and 272-A/K, Kpn
Questions trying to answer -

- 272-A/K was derived from 272-3 by ApaI + KpnI digestion
- Resulting DOM should have 2 fragments at 2452, 2526, 4271 [illegible] missing
Only 2 bands present
But of note 2526 was actually, but present weakly
& about as expected
Remember this is a mixture of 2 DNAs

272-3 KpnI
272-A/K KpnI
A
B
C

Band 3 is missing
CB/C & B/C
Band 3 is missing
Expected Weak

- NcoI digestion is expected to release a 1.5kb fragment suspending deletion junction
- ApaI should not cut
- KpnI should not cut

[redacted]

[redacted]

Note restriction pattern of 272-3 backbone is correct

272-3
250 A-C
NcoI
250 A-C

NcoI cuts as expected
RI/HindIII as expected

Start mapping up of A-
1) Sequence junction
2) Restrict CJ136-5-14-13
3) Fragment shot ~~236~~ REPLICON #7
272-dA 19

250, A-C
RI/HindIII
272, 250 A-C
ApaI

[redacted]

5:30 — Service Verified 6/1/93

Start ϕ of 293-RA & GT236 - two aperture

Purge culture 1 - Slot 8 (Rack Lin.) GT2284 #1

ϕ #1 grew - 75% ϕ

#2 did not grow

5:31

Re-spin DOA from both cultures, 1 ml

Re-suspend in pellet - 5 µl H2O

6:45 Put ϕ-mix 6 gel

Running 1% Agarose Gel

Running rec - 7th marker

5:00

Rec ϕ-DOA

150 µl Buffer - 3 ml

95 10 min

125 20 min 55°C

to 70°C, cool slowly to RmT

			(6:41)
16 µl 10x TH Pol buffer	-	30	30
ddH2O to 50µl	10 µl	40	30
ATP to 800µM	8 µl	48	2.4 µl gs. 222
95 to 94 µl	46	94	2.4 µl

1 µl TH Pol

2 µl DOA ligase

RmT - 5'

bl/c-2hr 4:30-6:30

RmT - ~~5~~ 3hrs

~~R 37°C two hours~~ 37°C 30'

EDTA to 25mM

6:42 Transform JM105 TB101 with 5 µl

Numerous colonies on both plates both ~ 1:2

Put #1 > 2x #2.

6-3-93 7:30 a.m.

Pick 12 colonies for mini preps

Negatives - some wt others only vector

6-4-93

Twelve more picked - all negative

6-5-93

Pick remaining colonies & cut

to page 18

Preparation of Reagents 6-3-93

3ug Digest 1.0 ml with 20 µl Cu 1

3 µl Cu + 1.5 ml H2O 1

Quick for completion of digest - complete

2.5µl 1M tris pH 8.3
2µl EtP
42°C, 30'
Read
Ethan

Kjeldahl digest

3µl H2O
5µl 10x Medium Salt
5µl 5mM diTP
2µl Kjeldahl
20', 37°C
~~20µl 10x~~
EDTA to 25mM
Add Chromatography Wtr to 50µl
Span column
Ethanol
Add 600µl H2O

OD240 = .08 = 5µg/ml Stat 96, 20°C. 6-2-93
OK by AGE.
No background in transformation of 6-5-93
Extracted DNA prepared again.

from pg 9

Quantitation of RVFM

Ligation

	Vector	Insert	10B	10x	Lig
Insert					
Vector	5	0	3	20	2
Insert only	0	5	3	20	2
+Repair	5	5	3	15	2
			(3)	(15)	2

(via 9) Rx T 200

1 hr 37°C 6-8-93

Contaminated

6 Tube Sine cells (pm)

Shown
Many fragments, one per fragment
None from left tube

Pick 200 of each for sequencing

RVF
RxRVF

Re-do fragment purification
① Ecori, Sal digestion 80 100µl of pT9-7 +Bg
② ~~Hind III digest~~
③ Final digestion
Repeated 6-8-93
④ T4 pol repair
Ecori precipitation 10ug of 1.3mg/ml .858ug/ml in 100µl
⑤ Lig + transform

Repeated 6-8-93 -- Purified by electrode Tube

Ecori 2x
Hind 20 Sal 53 RVF Blunt RVFSal Pmc

Ligation

	Vector	Ins	H2O	10x	Lig
Insert	0	5	20	3	2
RVFSal	5	5	15	3	2
	✓	✓	✓	✓	✓

9/06-9-93

PREPARATION OF LASER Wafers for Replicas

LS56-7A (Hab 7)

Pig skin - Ear Ref - Dan 71, Split-Bar 41 - ATS - Rep-Rj

30 yrs old 2 DPL 2 Ear

2 Que DOA

5 Que 10x 4

20 Que H_2O

2 Sub Kpn 1

2 Sub Bau H1 4/00

- Rem of extract
- Shot pot
- Trypsinize 3 Que 10x
  5 Sub 37°
  Pancho Lyse 24
- Select - 4 7 K6 fragments - 6-3-93
- Stud in Shot 24, 6-26 freezer unit

Ligation to replicas
to on page 12.
Include reactions with fragments so
may be significant parent vector contamination

Sample	Sus	Vector	10x	H_2O	Lig
LSK	3	0	3	22	2
LSK+Rep	3	3	3	17	2
	✓	✓	✓	✓	✓

Nick 9:00 a.m. 6-3-93
Add to JM 105 6-4-93
Contaminated
Add to succ cells 6-5-93 (2 ml) - Grew very small maybe
No colonies from replica sample ... doesn't necessarily
Numerous from purified fragment ... make sense

Pick 2 of each
N
Left N. - none to pick
Prepare additional fragment

to page 14

Preparation of O fragment
① Digest in H/Buffer as on previous page
0/N 37°C
② EtOH precipitate
③ Digest with BglI / BH
④ Phenol : EtOH
⑤ T4 Repair - Tris 10x
Sucr 1mM DTP
38 uL H_2O
1.5 µL T4 Pol
37°C 20'
2 µL EDTA
⑥ AGE elute

Fragment purified
Gel badly overloaded
Collected largest fragment
Frozen in slot 49

	Vector	Control
5 µL Insert		5
5 µL plasmid		0
3 µL 10x		3
15 µL H_2O		20
2 µL ligase		2

Replicon Tube 96 (6-2-92)

No fragment control - Why not?!

No colonies on replicon control
2/12 on N+ Replicon plate
All picked for mini preps
Digest with ApaI - stored minus
~1800 bp frag 30°C in H buffer
None predictive
Repeat fragment isolation.
Ligation - include pT218 Hinc2 as control

Sample	Vector	Insert	10x	H_2O	ligase	Cocktail
Sa Frag alone	0	5	3	20	2	10x 15
N+ Rep	2	5	3	18	2	H_2O 90
N+pT2	2	5	3	18	2	Lig 10
Rep alone	2	0	3	20	2	

T. Pole 1/4

Helper preparation continued.

In order to ensure CJ2236 are as they should be
Streak culture on AMP/CAM media. -OK
and inoculate into AMP/CAM broth. -yes

Purified & phenol extracted 6-9-93
Stored at -20°C ~~aliquot #3~~ slot 50
272-dA Lu Φ DOA

6-14-93
After standard technique and into ~~JM109~~ HB101
remainder of ~~slot 50~~ material treated
with ExoIII
in 50μl reaction
5μl 10x ExoIII
39μl DOA
5μl H_2O
1μl ExoIII 1.3μl BRL 76U/μl
5μl sample taken at start
5μl at finish
2μl. EDTA
q.s. 200μl
~~EtOH~~
~~EtOH~~
EtOH
Suspended in 40μl H_2O

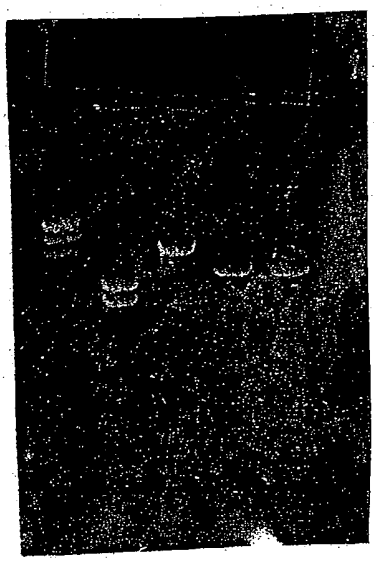
Only detectable difference was an increase in the amount of the faster migrating materials.

6-16-93
20 colonies picked and mini prep'd.
Only one recombined expected product ~ 3900bp R1/H3
only #19

frag. Redo mapping
R1/H3
R1/Nco - Where is second cut?
Pst/H3
Pst.

Grow additional O/N cultures for more mapping & sequence.

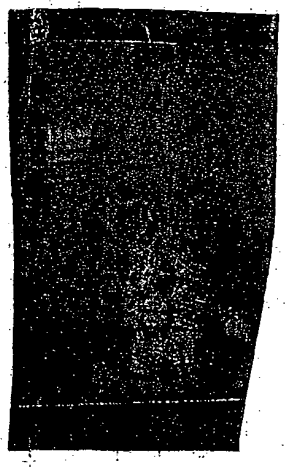
~~Additional question~~
~~to this cut~~
Pst-H3 cut can be explained if Pst from polylinker was transferred with clone.



(over)

Additional mapping
- Does 272-eA hook Pst site transfused with polylinker ?
- Where is NC01 site predicted
- Need R1/Pst
H3/Pst in high sets.

Identical pattern — H3/Pst, R1/Pst



SEQUENCE SHOWS EEE 265 CONTAMINANT.

6-22-93
ALTERNATIVE STRATEGY

ADDITION OF LEADER BY SEQUENTIAL PCR USING 3' DIFFERENT 5' PRIMERS
See PAGE 21

RVF – Replicon from page 12

Transformation of 6-10
Insert preparation producing too many colonies

∴ Insert fragment prep with PvuI and repurify fragment.

New vector prep
ClaI – CIP – Klenow 19.8 ug/ml #67

6-11-93

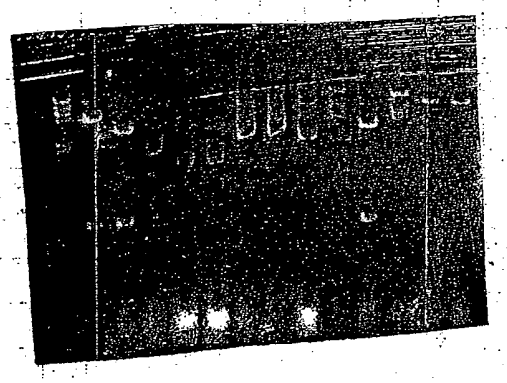
Ligations	Vector	Insert	10x	H_2O	Lig	Colonies
Vector	2	0	3	23 ✓	2	0-15
Insert	0	5	3	20 ✓	2	~30 colonies
+	2	5	3	18.	2	11 colonies
		✓	12 ✓	72 ✓	8	

RmT 6-11-93 6:45 pm —
6-14-93 Sh to DH5αF'

Picked 8 colonies R-1 — 8
Also pick two from Insert control C-1, C-2. – 9, 10
" " Vector V-1 V-2 11, 12

Digest with NheI as shown on page 8
Include RVF from Collett on control #59

Vector Controls – Replicon As Predicted
Insert controls
① Simple one – deletion of upstream PvuI fragment, vector snaked back



Why is replicon not taking fragments?
Not blunted properly?
T4?
Klenow?
Just vector for ability to close on itself. W/ & w/o PDK treatment.
2 reactions:
5 ul ~100 ng vector
1 – M Tris – DTT – ATP 2 ul

b/

Kinase of vector

Tube 57 1.8mg/ml

Sample	Vector	10x PNK	10mM ATP	H_2O	PNK
+	5	3	3	18	1
−	5	3	3	19	0

37°C - 30' ✓

ΔH 15' ✓

Ligation

~~Incub~~

	Plasmid	10x Lig	H_2O	Lig
+	3	3	22	2
−	3	3	22	2

Rm T - 0/0. ✓ ✓ ✓ ✓

~~Into same cells.~~

BUST! SHITTY CELLS!

PREPARE NEW 10S's 6-20-93

To PAGE 19

from 18

6-20-93
Use of Shuttle Tri Insert RVF & Lassa into Replicon. — Don's Suggestion

1- Inserts into shuttle vector, use Cla I sites to insert into Replicon.
Using shuttle 2
Lassa LS55160-7A digested with BamHI & EcoRI
& Bgl I Vector #40

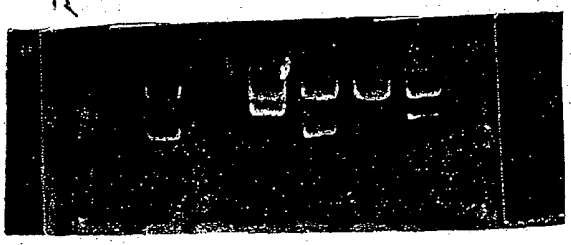
RVF pT7-7 digested with Sal I & EcoRI
Pvu I
Vector #54

ACE
Gene cocon 1 - Slot 97
Electrocute RVF Frags. Slot 98

Ligations

Sample	Vector	Insert	10x	H2O	Lig	Clones
B/2 vector #40	4 µl	0	3	21	2	0
LSN	4	5	3	16	2	20
Rmt-D/W 6-20-93	1					
S/R Vector #54	3	0	3	22	2	50
10-4-93 / RVF #1 + 54	3	5	3	17	2	>300
RVF FRAG	10	5	5	20	2	
	2	1	x	✓		

Purified RVF Frag
1-5 Cla I digested Lig.



2ug into Diff Sc
LSN's pT219 into Newest JM105's.

6-23
Cla digestion of 9 RVF-clones

#4,7 possibles
- Digest #4 w/ R1 & Sal I
- Digest remainder of mini for Cla I frag purification

Purified as RVF-Shuttle 2
Slot 9 Replicon Box
Should have reintroduced cut to verify vector insert layout.

Purified RVF-M Shuttle 2 Cla Frag
Slot 100, -20°C

Repeated (9-24-93)

6-23-93

- 12 colonies of LSN in Stuttle 2 picked
  ≈ minis.
- Digest w/ ClaI or BamHI/EcoRI

OF FIRST 7, 5 are positives

- Digest #2 with XbaI to assure orientation - should give 4.5kbp frag
  Digest remainder w/ ClaI / Freeze in Slot 10
  and Gene Clean

#2 Frozen in Slot
10 of Replicon Box

1-7 ClaI
1-7 BamHI
ran gel frag.

Ligations

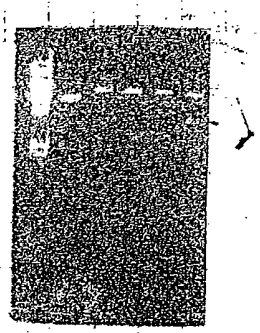
	Vector	Insert	10x	H2O	Lig	# Colonies
Replicon	2	0	3	23	2	0
RSF	2	5	3	18	2	2
LASSA	2	5	3	18	2	3
	✓		✓	✓	✓	

Rm T, O/N

- Into JM105 6-24-93
- 6-25

All five colonies picked.
3 N, 2 RSF
1-3 4,5

Clas digested
N-2, N-3 + → lanes 3,4,6
RSF-2 +



Helper Construction by Sequential PCR.

PCR to fuse oligo Helper 1 to structural gene.

PCR

Non phosphorylated primers

- Helper 1 3μM 5.5μl

~~Replicase~~

Help Replicase 72μM 2.7μl

DOA - Putting substrate #1 prepared previously

H_2O	53.3	53.3	53.3	53.3	51.3	256.5 ✓	
10x buffer	10	10	10	10	10	143	50 ✓
	8	8	8	8	8	143	40 ✓
Primers							
Helper 1	5.5	5.5	5.5	5.5	77.5	27.5 ✓	
Help Replicase	2.7	2.7	2.7	2.7		13.5 ✓	82
Taq	0.5	0.5	0.5	0.5	78	✓	
$MgCl_2$ (25mM)	4	8	10	12		✓	
DOA	10	10	10	10			

Separate file 53

6-25-93

10μl of each onto AGE

↳ negative all across

Repeat using VEE cDNA.

Repeat ~~6-30-93~~ 7-2

~~Substrate~~ p2-20

272-AA19

Dilute plasmid to 1mg, 100ng, 10ng, 1ng/ml,

Start with intact plasmid. take 4b

1.8 mg/ml stock

Dilute Ⓐ 1:18 = 100ng/ml

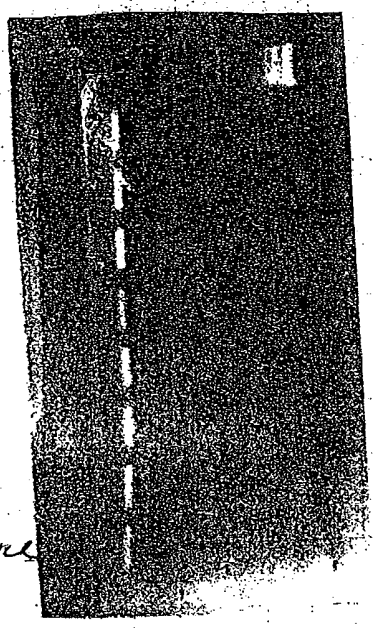
Ⓑ 1:10 10ng/ml

Ⓒ 1:10 1.0ng

Ⓓ 1:10 100ng/ml — Ⓔ 1:10 (10ng/ml) — Ⓕ 1:10 (1ng/ml) — Ⓖ 1:10 100pg/ml

Repeated
9-5

H_2O	61.4 ✓	1 =	100ng ✓ – 10µl B
10x	10 ✓	2	10ng ✓ – 10µl C
dNTP	8 ✓	3	1ng ✓ – 10µl D
Primers		4	100pg – 10µl E
Repl (24.3)	2.7 ✓	5	10pg – 10µl F
Help Primer (11.6)	1.4	6	1pg – 10µl G
Tag (0.5)	0.5	7	0.1pg – 10µl H
$MgCl_2$	6	8	0.01pg – 10µl I
DOA	10	10	10



Band appeared in each lane

Synthesize oligo helper 2

5' GCG CAT GAG AGA AGC CCA GAC CAA TTA CC
TAC 3'

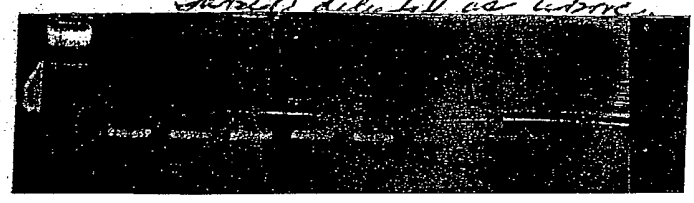
Should have been T → Helper 2A oligo
Slot C4

Discard reactions 1–6
Save reactions 7 & 8 at -20
labelled Helper1 PCR until saved

1-11-93 Oligo is ready. Prepared QD 7-11-93
Dry stored QD over hum 56°C
Series of dilutions again Diluted QD

Tube B		H_2O	67.1	67 –
A 1:10		10x	10	100 ✓
B 1:100	5µl of each	dNTP	8	80 ✓
C 1:1000		Primer		
D 1:10000	4µM	Helper 2	2	20 ✓
E 1:10 5		Helper 1	1.4	14 ✓
F 1:10 6		Tag	0.5	5 ✓
G 1:10 7	2mM	$MgCl_2$	6 / 17.9	60 ✓
H 1:10 8				
I 1:10 9		DOA	10	

Tube B diluted as above.



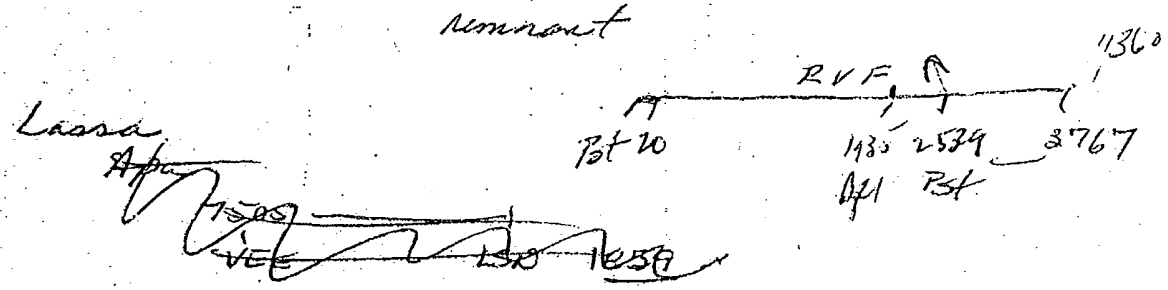
Put 1 tube in Nov. PCR 6pg

From page 30

Mapping Repliron Constructs

Do not use HindIII - found in remaining polylinker

RVF
Pst digestion should yield (lots RVF+VSV39) + remnant of vector
4461 — ~7562 — 10003
3Kb MCS 2.5Kb RVF
remnant



Pst ~1200bp
Pst of MCS 1170 of LSV

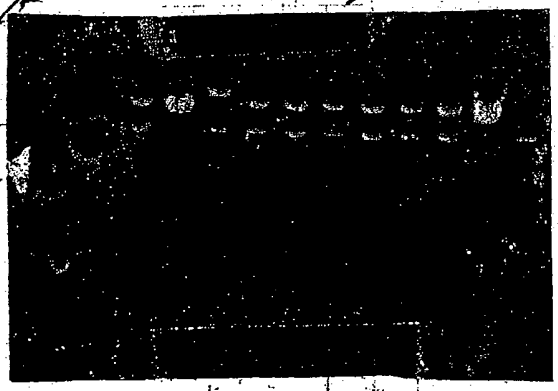
Primer Map Page 152

Xba —— Xba needs to be repeated w/ shuttle construct
415

Digest N2, -3 & 2-2 with Pst.
Include Repliron as control.

6-28
10 additional colonies (RVF) of transformed DH5α picked

6-29
Digested with Cla
Fractions 1, 3, 4, 5, 6, 7, 8



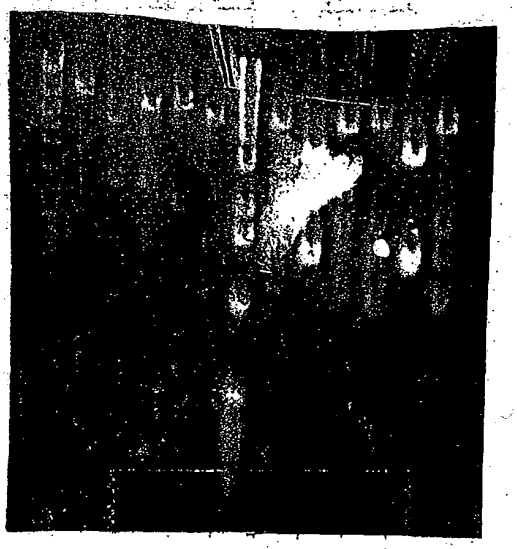
Digest with PstI
#2, 10 negative
1 - positive
3 - positive
Grow 1, 2, 3 for sequencing

Additional maps ApaI — BglI digest in Buffer 4 - 30°C
1, 2, 3 BglI - SphI
Repliron
Also cut repliron of each alone

ApaI 7505
BglI 7499
SphI 11414

Start O/N cultures for sequence

7-9-93



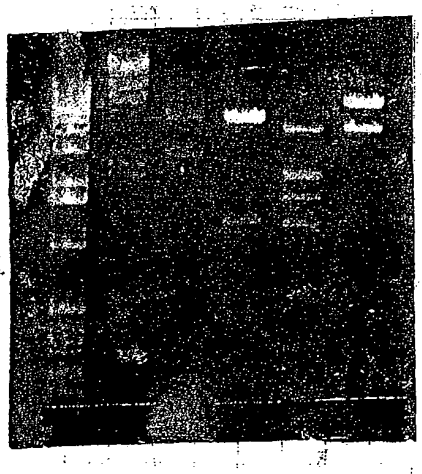
1
Replicon ApaI
ApaI / BglI
BglI
SphI
BglI / SphI
KB ladder
R1-R3
ApaI / BglI
R1-R3
BglI / SphI

Loss of 1 : 3

Second 2

Further mapping of Shuttle 2-RVF

PstI digestion
~~Shuttle~~ Release
2.5 Kb frag from
shuttle PstI site to
2593 of RVF M.
Release fragment
is only ~ 1500 nt.



Kb
λ
~~CHELG~~ StyI
StyI - CHELG
PstI RVF/Rep
RI/BglI
RI/SalI

Redo check of
Shuttle 2-RVF
#9, Replicon BX
Also redo ligation
of RVF Cla frag into replicon

7-10-93

Sample	Vector		Insert	ddH	H_2O	Lig	
RVF-Replicon	9	2	5 (166)	3	18	2	12:15
RVF-Shuttle 2	54	3	5 (98)	3	17	2	

No Colonies on 10-5
Info DH5 7-12-93

Topoisomerase

Replicon cassette in Bam → R1 site from shuttle 2

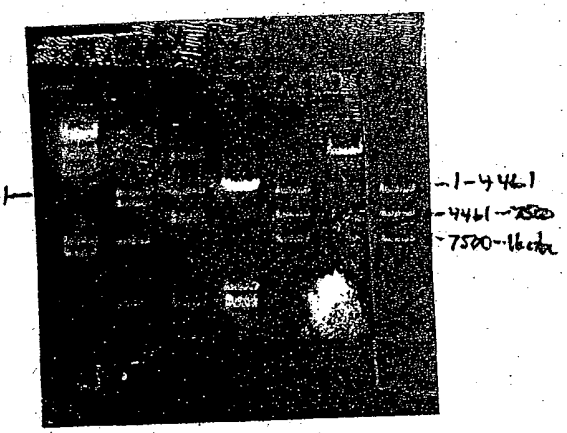
6/29 picked 12 colonies grew up mini preps

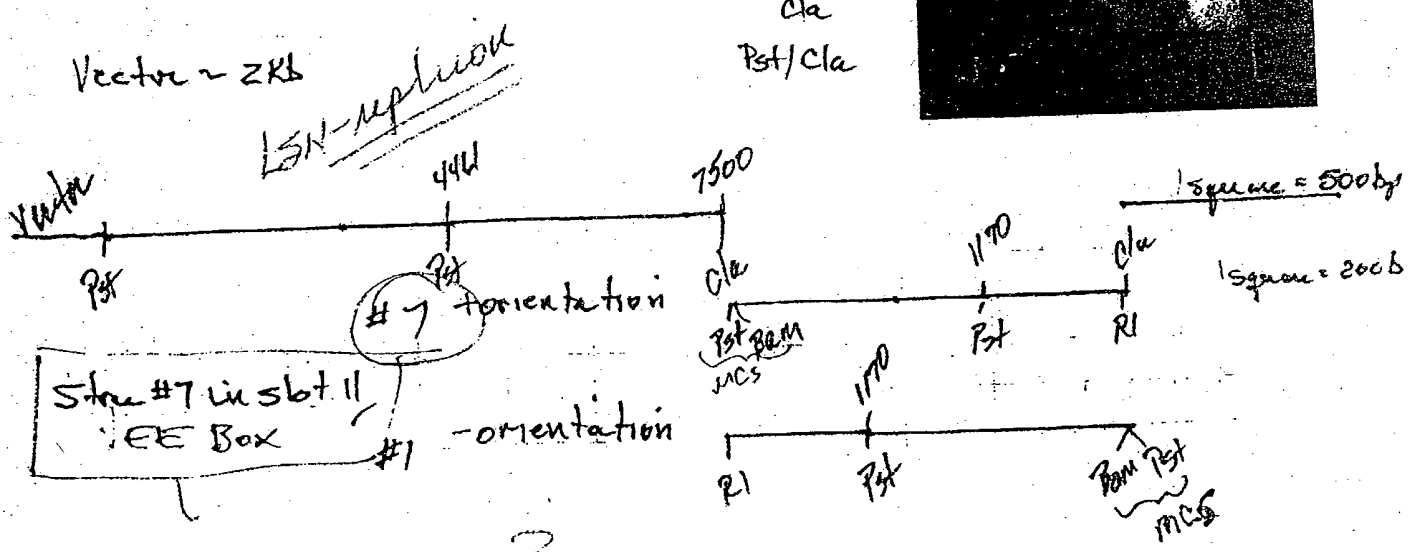
6/30 Mini preps - DNA isolated
Cut ε ClaI
cocktail 10x react 1 22 µl
ClaI 2 µl
H2O 196 µl
17 µl/tube + 3 µl DNA
1 hr digest 37°C
run on gel.

#1, 7, 10 positive for Insert.

1, 7, 10 cut with Pst
Should release ~1200 bp fragment - LSN 1170 ?
remnant of MCS of shuttle.
└ No - Because relative position of two sites remains regardless of orientation

Mapping Orientation





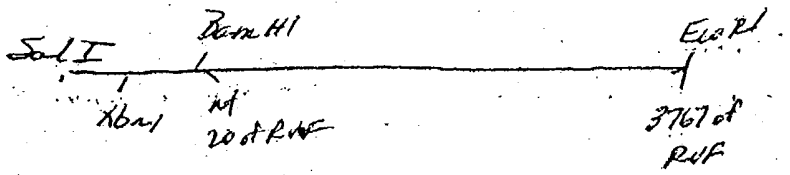
9-24

RVF Replicon Ligation

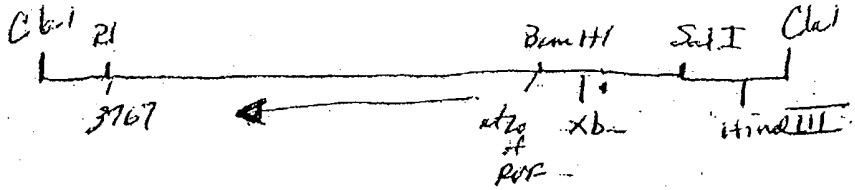
Sample	X Vector	X Ins	X 10×	X H_2O	Lig
Replicon	2	0	3	23	2
RVF+Rep	2	5	3	18	2
RVF frag	0	5	3	20	2

Repeat of cloning

10-5-93 Prepare Sal I / RI frag from 77-7
Insert into Sal-I / RI Shuttle 2
Into Sure Shots 10-5-93
At this point the fragment maps is:



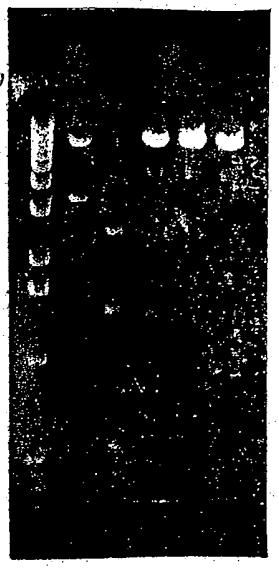
In shuttle 2



Transformation Results
Shuttle alone — > 500 colonies
Frag alone
Appears to be contaminated
Redo

10-14-93

6 colonies picked ClaI digest
#1 - ~3.7 KB + > 6 KB
Digest with BamHI
HindIII
PstI
Is it RVF?



No - Discard

To Page 75

from page 22

Helper Preparation cont'd.

Use of oligo - Helper3 - to add last of leader sequence.

Utilizing Helper 2 PCR as substrate.

Dilute reaction 2 tube # 6 over a series of 10x dilutions as on page 22.

- Set file 53 PCR reaction as before w/ 30 cycles.

7-4-93

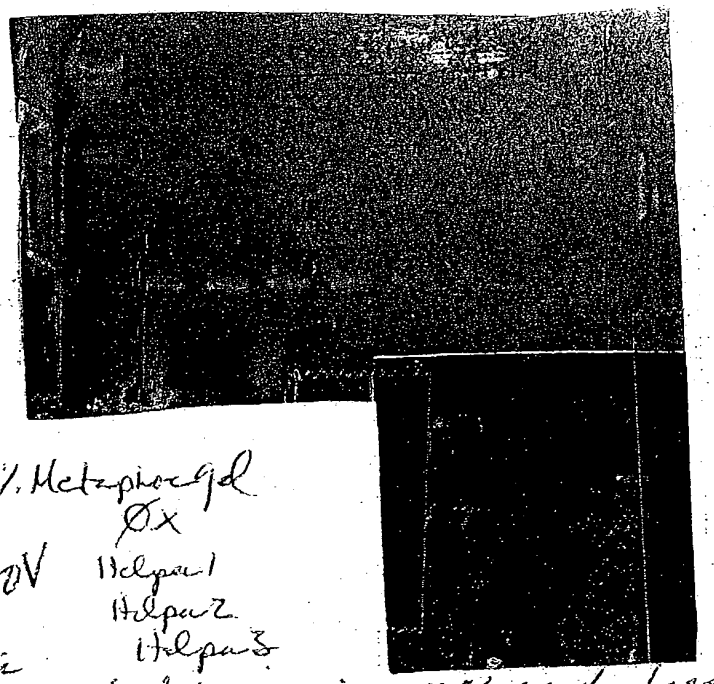
PCR

	54!
H_2O	~~50~~ 80.1
10x	10µl
dNTP	8
Premix	
HelpRev	1.4
Helper3	10µl
Taq	0.5
$MgCl_2$	6
DDA	10

Repeat 10 cocktails

file 53 - Final F 0.5µM

Positive reaction in each sample



3% Metaphor gel
ØX

Helper Assembly 1hr/100V Helper1
Helper2
Helper3

① Vector Preparation
digest 272-3 w/ XbaI Dot - purify 2kb vector frag
Done & held at -20°

② Structural proteins - harvest ~4Kb Cvn — Dot frag
Include Hind3 on record
preparation 5-6-93

③ Initiation
XbaI - Cvn digest of Helper 3 - Pick frag

7-16

Check restriction maps of 272-3

① digest w/ Xba - linear

② Cvn - releases 4Kb frag nt 3500 - 7500 (C)

③ Not - linear

Computer predicts an Xba fragment should be released.

Run 1ug of Cvn-1-Not digest - no 4Kb fragment
Predicted 4Kb, 4Kb, 5Kb slot 4B -20°C

no 3.7Kb

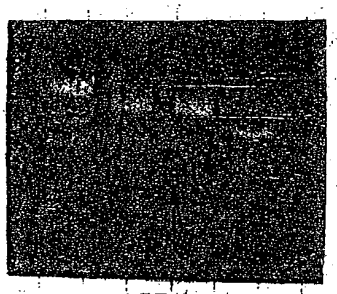
8-10-93

7-18-93 Purify 2Kb Xba - Not frag no vector.

As predicted

How to clean up the frag I want?

272-3
Xba 1
Not 1
Cvn 1

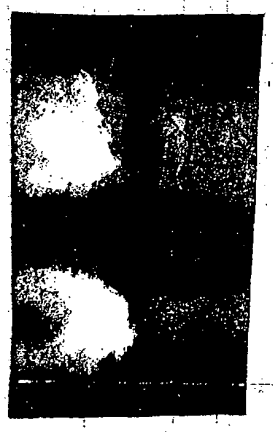


Digest with 15ug ea Pvu1 after adding 40ul high salt buffer.

5ul to 0.7% gel

7-19-93

Xba - Not 1 gene cleaned frag in slot 8-20 Discarded



4Kb fragment isolated on 0.7% gel.
Purify by electroelution

Cvn - Xba cut PCR product isolated by gene clean 5hrs - 20°, slot 4/5 Discarded

7-21-93 Cvn - Not 1 3Kb frag gene cleaned 5hrs - 20° slot 4/5

Ligation
5ul Cvn - Xba
5ul Xba - Not 1
5ul Cvn - Not 1
2ul 10
5ul H2O

Run 1/2 Discarded

from 28

1-20

PCR product may have been lost during Gene Cleaning
∴ Repeated by same protocol as before

Reaction is positive
- Remove oil on parafilm
- Elect ppt.
Suspend in 30 µl H_2O

Hyb in Med-High
Cvn

Hyb in Med-High
Xba

Remainder adjust to med-high
Xba - Cvn

Mini gel to determine cutting pairs to use.

1-23-93

Run new [illegible] agarose
Electrophoresis
- 70°C, clear
Repeat
Rinse
Dry
Suspend in 30 µl H_2O load 2 electroelutions)
Store at -20, elute 45 (Discarded)

Running approx gel of all three digs.

Xb lad

Xb ladder

#8 5' terminus Xba - Cvn - present
45 Stanford - Cvn - Dot
48 Nutra - Dot - Xba

Ligation
45 10x = 3 ✓ H_2O 15 ✓
15 - 5 Ligase 7A-4K ✓
45 - 3 ✓
48 - 3 ✓

7-14 Prior to ligation, a sample of each DNA was run on AGE and stained.

It appears that the Cvn I - Not I fragment is actually a closely spaced doublet.
Xba-Cvn fragment was present but not visible in photo

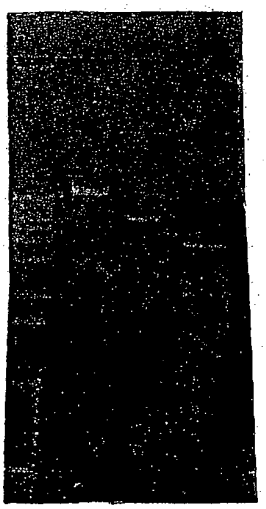
7-15 Transformation of DH5α cells yielded several hundred colonies. 12 picked for restriction digestion

1Kb
Xba-Cvn
Cvn-Not
Not-Xba

7-16 Minipreps
digested with Xba
and R1 + Hind3

#1 has appropriate digest pattern; only one of 12

Digested w/
Cvn I / Not I
R1 / Not I
Xba I
Hind 3 / Not I
Xba / Not I
Xba / Cvn I



Cvn I / Not I
R1 / Not I
Xba I
Hind 3 / Not I
Xba / Not I
Xba / Cvn I

Freezer #1 Helper 1 VEC Box 2 Row 12
Minipreps Stored 7-16-93
DNA stored in slot 61

Recorded on page 43
Page 39

Nested Helpers.

Primer to generate 5' nested helpers, 47G from 105 gene, stored to remind

-. Oligo Helper 1 was prepared, washed ATG is exchanged for a T.

↑

Then A ligated 5' end by PCR with Rsp5 helper 2A + Helper 3

Used new primer located just downstream of Rsr2 restriction primer.

Rsr2 nick 47 5'G CGGACCG

Negative primer - Helper Rsr

TCTAACTCCC TAATTGGCTT GGTG

In order to sequence across fusion junction, a sequencing primer was also synthesized which will allow sequencing upstream from Rsr2 initiator.

Feb 3-6-94

Start all primed PCR again 1 mM

(A) Helper 1 + Rsr2 primer pairs - Synthesis ++

Dilute tube 3 1000 fold

(B) Primers 2A + Rsr2 Mg Cl2 1, 1.5, 2, 2.5

Dilute tube 2 1000 fold tubes 2, 3, 4 ++

(C) Primers Helper 3 + Rsr 2

dilute

EACH crude rxn diluted and stored in slot (10 V)

7/30/93 Helper 2nd Generation
- using Helper 2A

Repeated 5-25-93

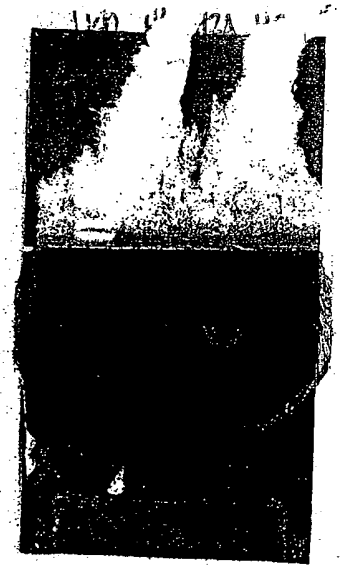
59.1 µL H_2O
10 µL 10x PCR soln II
8 µL dNTPs
5 µL Helper 2A (21µM) ~~C4~~
1.4 µL Helper reverse tube A3
6 µL 25mM $MgCl_2$
10 µL of 1:100 diluted Helper 1 (tube 8) PCR product

.5µL Taq(P) ←

Mineral oil
30 cycles (94°-1'; 52°-2'; 72°-2')

PCR positive Bright band @ 400bp Stored -20°C freezer

Use of Helper 3 to add last of leader sequence
Diluted H-2A 1:100 for use in next PCR rxn.

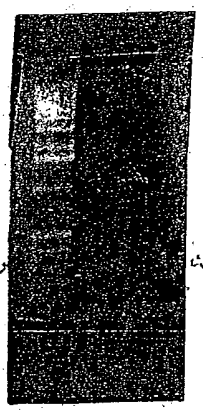


✓54.1µL H_2O ✓8-25
10.0µL 10x PCR soln II ✓
8.0µL dNTPs ✓
1.4µL Helper rev A3 ✓
10.0µL Helper 3 G3 ✓
.5µL Taq(P) ✓
6µL MgCl (25mM) ✓
10µL dilute H-2A PCR product 1:100

Mineral oil, 30 cycles (94-1', 52°-2', 72°-2') to page 51

synthesis fine.

7/31/93 H3A
- Removed oil on parafilm
- EtOH ppt (10µL NH_4Ac + 1mL EtOH)
- Resuspended in 30µL H_2O
  Cut w XbaI & CvnI in Med-high buffer
- 3µL on mini gel to check
- Ran rest out on midsize. Mike
  Cut out band, stored @ 4°C.



Stock slot 57 Helper 3A

8-1-93

From 32.

Ligation of Helper 2

✓ 3µl 10x ligase
✓ 5µl Cvn1 – Not 1 Frag Slot 48
✓ 5µl Xba1 – Not 1 Vector Slot #8
✓ 5µl H3A Cvn1 – Xba1 Frag Slot #57
✓ 10µl H_2O
✓ 2µl ligase

Into Tuc shot cells. 8-2-93

12 colonies picked
Digested with Xba1/Not1

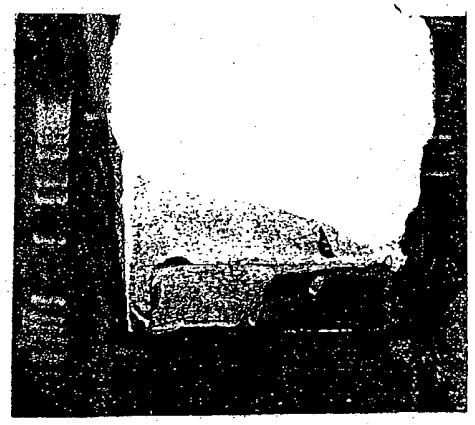
Kb
1-12
Xba1/Not

#5 1, 5, 8, 10, 11, 12
Digest with Cvn1

#5
Cvn1 digests Xba/Not digests
1, 5, 9, 10, 11, 12 10, 11

Verify Cvn-Xba Insertion by PCR on Helper 2A & Helper Constructs.

8-25
Repeatedly negative.
∴ Redo preparation of PCR Frag as indicated in previous page



3/1/93 Helper d1 construction by sequential PCR using DEEPVENT

Template 272-3
986 µg/mL

786 µg/mL (X µL) = (100 µg/mL)(10 µL)
X = 1.3
8.7 µL H2O

4 = 100 µg/mL
3 = 10 µg/mL → 1
2 = 1 µg/mL → 2
1 = 100 ng/mL → 3
= 10 ng/mL → 4
= 1 ng/mL → 5
= 100 pg/mL → 6
= 10 pg/mL → 7
= 1 pg/mL

Tubes:	A	B	C	D
H2O	65.5	57.5	53.5	49.5
10x	10	10	10	10
(50mM) MgSO4	0	8	12	16
BSA	.5	.5	.5	.5
(10mM) dNTP	3	3	3	3
template	10	10	10	10
primers	5+5	5+5	5+5	5+5
DEXO	1	1	1	1

al Mg is:
mM Mg
mM N
mM H

; 30 cycles (94°-1', 52°-1', 72°-1')
ing for bp fragment
lelper d1 & HelperSr II

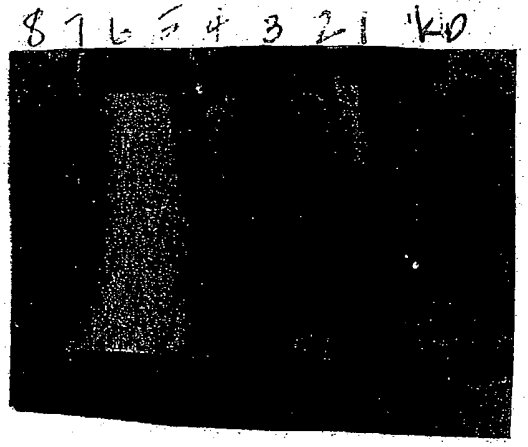
15

No evidence of synthesis in rest of samples.
ID used to serve as template @ [Mg]°C.

3B

go to pg 35

8/8/93 Helper dil Construction (cont.)



Serial dil q"ID"

1:10	→	1
1:100	→	2
1:1000	10µl of each	3
1:10⁴	→	4
1:10⁵	→	5
1:10⁶	→	6
1:10⁷	→	7
1:10⁸	→	8
1:10⁹	→	9

Cocktail
- 53.5µl H2O
- 10 µl 10x
- 12 µl MgSO4
- .5 µl BSA
- 3 µl dNTP
- 5 µl helper2A
- 5µl helper RSV
- 1 µl (P) Exo±

90 µl/tube + 10µl template
mineral oil 30 cycles (94°-1', 52°-1', 72°-1')
(File 5b)

Took sample #1 for next step

		B	C [Mg]
A:	Straight 10µl	1	2
B:	1:2 →	3	4
C:	1:5 →	5	6

B = 4mM
C = '

Cocktail
- 48.5µl H2O
- 10.0 µl 10x
- .5 µl BSA
- 3 µl dNTP
- 10 µl help3
- 5µl RSV
- 8 µl MgSO4
- 1 µl Pol

80µl/tube
30cycles; File 5b as above.

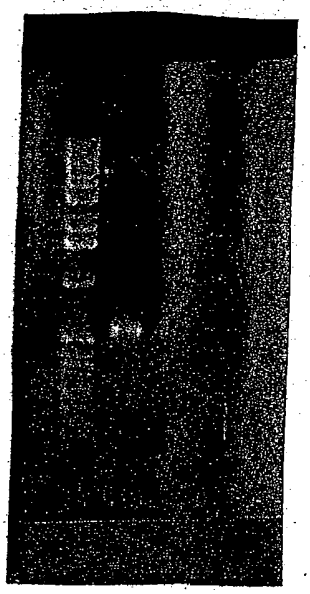
faint bands & smears in lanes.
redo @ lower [Mg]

-Saved samples 1-4. (Tossed by [illegible] yield on later rxn p. 21 → pg 39)

yr. 35

8/3/93 3rd sequential PCR helper d1 construction
no Mg added

60.5 µL H_2O
10 µL 10x
.5 µL BSA
3 µL dNTPs
10 µL sample (undiluted tube #1)
10 µL helper 3
5 µL rsr
1 µL DeepVent(P) Exo+
Oil, File 56 (94-1', 52-1', 72-1')
O/N.



-Also set up 2nd sequential PCR
helper 2A & rsr, since band faint

65.5 µL H_2O
10 µL 10x
.5 µL ~~BSA~~
3 µL dNTP
10 µL template (10uL straight from ID)
5 µL helper 2A
5 µL rsr
1 µL DeepVent(P) Exo+
Oil File 56 (94, 52, 72, -1' ea)
O/N

8/4/93 Since 3 not very good but 2A nice,
set up 3rd sequential rxn using sample 2A

60 µL H_2O
10 µL 10x
.5 µL BSA
3 µL dNTP
10 µL sample 2A (above)
10 µL helper 3
5 µL rsr
1 µL DeepVent Exo+

File 56

to gel 3

From 30

8-5-93

Helper 2 mapping cont'd

Still can't visualize Cm r-xba fragment.
∴ Continue restriction digestion

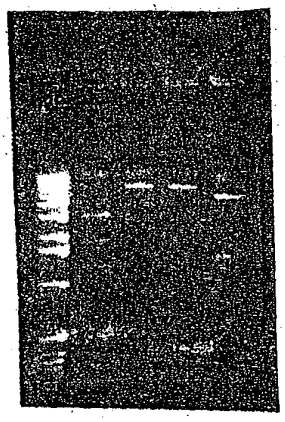
Pst I digestion predicted to release

at 39 — 1817 — 3045 — 3224 → 4001 vector
1778 1228 179 ← 2406 bp.

Hind 3/Nco → 1431 bp lane 5+

All fragments as predicted.

further verified by PCR with Helper 2A & Helper Reverse Oligo's



8/5/93 PCR verification
replicon colonies # 1,5,9,11,12
DNA diluted 1:10,000

H2O	59.1 µL
10X	10 µL (-Mg)
dNTP	8 µL
helper 2A	5 µL (20 µM 04)
helper rsr.	1.4 µL (F14) - "rev"
Taq (P)	.5 µL
25 mM MgCl2	6 µL
template	10 µL

30 cycles; File 55
(94-1', 52°-2', 72°2')

No evidence of synthesis. Maybe DNA too dilute.

8/6/93 Repeat PCR ε dilutions 1:10, 1:100, 1:1000/sample
as above. O/N
8/6 No evidence of syntheses.

8/6/93 Wrong primer should have used helper reverse & helper 2 instead of helper rsr & 2A. Repeated above ε dilutions of template 1:10, 1:100, 1:1000.
8/7 No evidence of syntheses. to p. 43

8/6/93

Transcription run off experiments ± HA41 ± helper 1

control 43 µL	helper 1 33 µL	HA41 24 µL	H_2O
		10 µL	10x Ecription rxn mix
		10 µL	ATP
		10 µL	CTP
		10 µL	GTP
		10 µL	TTP
		5 µL	^{32}P-UTP
control 2 µL	helper 1 12 µL	HA41 21 µL	template (1 µg)
		10 µL	Rz mix

4 hrs @ 37°C

→ forgot to take TCA samples, at end of 4 hrs took 2 samples, 1 TCA ppt

Counts.	1	2	Net	%
Control	40383	697	583	0.017
HA41	22527	6708	6594	29%
Helper 1	35904	114		

0-272 from 36

Helper d1 construction

Three PCR steps run in parallel lanes.

Digest 2.5ug pZ72-3 with RsrII
% in Low Salt — DH = buffer
① Is it cut once?
If so, cut with ApaI
but blunt end and close
→ Helper 3

Then - double dig vect with XbaI + RsrII
- insert Helper d1 PCR fragment.
Repeat RsrII ApaI digestion → Helper d1
→ Helper d2

9/93 Helper d1 construction
Took PCR product above & removed oil on parafilm. How to Direct or EtOH ppt
T4 blunt end: 10µL dNTP, 11µL 10X, 1µL T4 ®. 10' @ 37°C.
EtOH ppt. Resuspended pellet in 20µL ran out on gel. Cut out 600bp fragment, electroluted. EtOH.
Resuspended pellets in 15µL H2O, 2µL PNK 10X
2µL 10mM ATP, 1µL PNK, 37°C for 1 hour
Heat inactivated 65°C for 15'.
Ligation:
5µL fragment
3µL pIC19 HincII (20ng)
2µL 10X Ligase
2µL DNA Ligase
8µL H2O
O/N @ RT.

8/10/93 MP transformed into DH5α.

8/11/93 5 white colonies picked for mini preps

8/12/93 Mini preps done. Cut w/ XbaI & RsrII looking for 600bp fragment.
Set up RsrII digest first in low salt* 1½hr 37°C.
EtOH ppt (5µL NH4OAc + 1mL EtOH -70°, 30')
*Took a 2 or 3µL sample to run on gel RsrII alone. go to 4

Helper RSR construction

8/6/93 Cut 272-3 ε RSR, ran ½ out on a gel to
to save cut only once. Then digested ε ApaI
(EtOH after RSR resuspended for Apa digest)
Blunt end & ligated back to itself.
Blunt end: 50μl total
20μl DNA
5μl dNTP
5μl 10x T4 (P)
1μl T4 (P)
19μl H2O

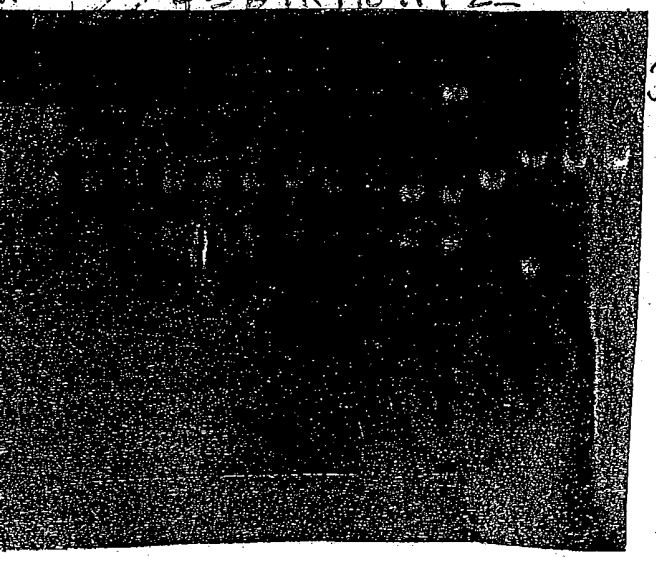
8/7 ran on gel electroluted & to ligated.
16μl H2O
2μl 10x ligase
2μl ligase (DNA)
O/N @ RT

8/8 T formed into JM105 - LB/AMP

8/9 Picked 12 colonies for mini's O/N growth

8/10 Mini preps cut ε EcoRI HindIII
~2000bp vector + ~2000 bp.
All but #12 positive
Cmosc #1 Tossed rest.
Glycerol stock (#13)
~70z. Maxi prep
growing

Cut #1 RSR ε XbaI & CVN
as verification
~5-6000bp & ~1000bs
fragment



8/10/93 Digest of 272-3 c SalI, BstEII, & SplI.

3μL 272-3 DNA

SalI: 2μL react 10, 1μL SalI, 14μL H_2O 37°C - 1½h

BstEII: 2μL react 2. 1μL BstEII, 14μL H_2O 60°C - 1½

SplI: 2μL 10x hi, 2μL 0.1% BSA, 1μL spl, 12μL H_2O, 55°C 1½hr.

ran on gel (see photo pg 40)
BstEII & SplI cut only once
will prepare helper BstEII & a helper SplI (double digest c ApaI) ligate back as c Helper Bst.

8/11/93 Construction Helper BstEII

5μL DNA, 2μL react 2, 1μL BstEII (20μL total) 60°C for 1½hrs.
Run 2μL on gel to check. EtOH ppt (5μL NH_4OAc + 1mL EtOH.
to set up ApaI digest (2μL react 4, 1μL ApaI, 17μL H_2O). Digest 1½ hrs. ran out on gel.
Appears some didn't cut
Took larger of "doublet" band
cut out ~528g
want ~ 771? "extracted" from gel
4°C O/N.

BstEII SplI

8/12/93 Electroeluted. EtOH.
Blunt end ligation:
Blunt end: 20μL total
15μL DNA
3μL dNTP
2μL 10x T4 (P)
1μL T4 (P) 37°-15' (EDTA 1μL to stop)
- should have blunt ended then ran on gel & electroluted. So will blunt end & extract, EtOH & then ligate

go to 47

8/11/93 Construction of Spe helper.

5μL 272-3, 2μL 10x high, 2μL 0.1% BSA, 1μL Spe
10μL H_2O. Cut 1½ hr. ran out on gel - fine.
5μL NH_4OAc + 1mL EtOH -70°C for 30'.
Resuspended in 17μL H_2O, 2μL react 4, 1μL ApaI
Digested 1½ hrs. ran out on gel.
Sec gel p 4-1 - Took lger band
Cut out ~355' Want ~10kb band = extracted
from gel, 4°C O/N.

8/12/93 Electroeluted bands. EtOH.
- Should have blunt ended then run on gel
& electroeluted. So will blunt-end,
Ø extract & EtOH ppt, to ligate.

Blunt end:
15μL H_2O resusp DNA
2μL dNTP
2μL 10x T4 (P)
1μL T4 (P).
37°C for 15'
1μL EDTA, Ø extract, EtOH - 70°C, 30'.

Ligation: 16μL H_2O (resusp)
2μL 10x ligase
2μL DNA ligase
O/N @ RT

8/13/93 Formation of JM105s & Helper Spe ligation
8/14/93 No colonies

8/16/93 Repeat Spe-Apa digestion above.
Didn't cut NO spe left.

8/17/93 Formed ligation from 8/12/93 into
Sure shot cells. (see protocol p. 4-3)
Inc @ 0°C (should have been 30°).

8/18/93 No colonies

9/1/93 Cut 272-3 & Spe
5μL 272-3 (#55), 2μL 10x high, 2μL 0.1% BSA,
1μL Spe 55°, 1½ hrs.
1μL EDTA, Ø extract, EtOH
Resu in 17μL, 2μL react 4, 1μL Apa
30°C, ½ hrs.

9c to 59

8-11-93

Preparation of Helper 1 & 2 - Repeat

Verification of Helper 2 is not working
- have not been able to analyze Xba-Cm frag
PCR ~~test~~ negative.
Also 3.7Kb frag is contaminated.
∴ Repeat new 3.7Kb frag

Redo ligation

Sample #	3.7 (8/8)	Vector #8	Cm-Rot	10x	H2O	Ligase
A-Helper 1	5	5	5µl #45 X	3	15	2
B-Helper 2	5 ✓	5 x	5 #57 X ✓	3 ✓	15 ✓	2 ✓

✓ 8/12/93 Transformation of JM105s (37°C O/N) LB/AMP
8/13 No colonies repeated 8/13.

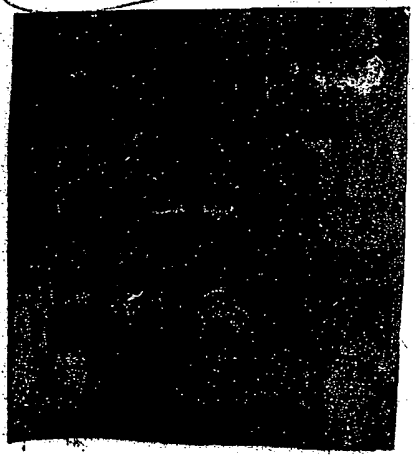
8/14/93 No colonies ??

~~8/16/93 Repeat Spe-Apa #~~

8/17/93 Transformed into Sure Shot cells.
Thawed cells on ice. Added 2µl β-ME
1µl ligation mix on ice ~1hr. (should have been 30' - forgot), 42°C for 45sec
on ice 2'. Added 45µl SOC. Shook 1hr.
plated 150µL/plate Xgal plates (3plates/transf.)
Overnight 37°C

8/18/93 No colonies. left in incubator.

8/19/93 2 colonies/helper. Mini preps set up.



8/23/93 Mini preps cut ε EcoRI-HindIII.
- looking for 4-5000 bp fragment
Cut 1-2 & 2-1 ε Cm & Xba
med-high salt & 1-1 & 2-2
No 3-400 bp fragment
Discarded all

1Kb
1-1
1-2
2-1
2-2

8/11/93 PCR primers (for "deletion" to achieve "RSR2 series")

Helper RSR-B (F) MW = 10,091.6 stored P-4; -20°
5' ATC GGA CCG AAG ATG TTC CGG TTC CAG CCA ATG 5'
540 µg/mL (in PCR use 19 µL)

V26Nco (R) MW = 7775.0
5' TCC GTG TTC TGG GGG ATG AGT ATA G 3'
354 µg/mL (in PCR use 2.2 µL)

8/11 PCR
Cocktail
57.5 µL H_2O
10 µL 10x (-Mg)
8 µL dNTPs
.5 µL Taq (P)
780 µL/tube

samples	H_2O	Mg (100mM)	template	primers [1µM] each	
C	0	4µL	EEE (1/1 very dilute)	ERP9A & B (Subteach)	
1	8.4	1.5µL	T	RSRB	V26Nco
2	6.9	3.0	JT23	1.9µL	2.2µL
3	8.9	4.0	(1:100)	1	1
			1		

Taq (P)

oil. File #55 (94-1'; 52-2'; 72-2')
* since didn't use deep vent need to blunt end tn kinase before ligation

#2 looks best, but not great.
-20°C, stored.

8/14/93 Repeated c Deep Vent.
Cocktail
55.4 µL H_2O
10 µL 10x
.5 µL BSA
3 µL dNTP
1.9 µL RSR-B
2.2 µL V26Nco
1 µL Deep Vent-Exo⁻
74 µL/tube

Cpt 1
DG

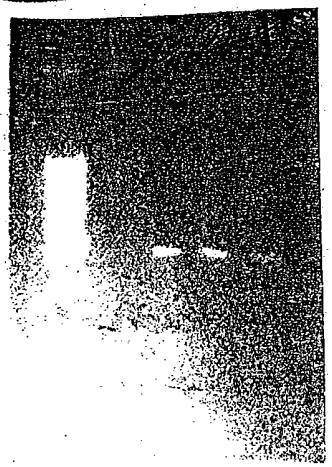
DeepVent PCR ± Helper RSRB & V26NCO

tubes	1	2	3	4
H_2O	16	8	4	0
Mg	0	8	12	16
272-3 (1:100)	10	10	10	10

Oil, 30 cycles (94-1', 52°-1', 72°-2') O/N

Ran on mini gel O/N. (Well #1 hole so sample leaked out)

5/15/93 All lanes showed synthesis ~~of~~ in #1 w/ leaked out.



Took #2, removed oil on parafilm
Took a 10µL aliquot for ligation into pTZ19mmcII
Added DNA loading buffer to both samples (one & rest)
T4, Ran on gel electroelute + ppt O/N.

5/16/93
Resuspended 10µL aliquot in 15µL H_2O
2µL 10xPNK, 2µL ATP, 1µL PNK.
37°C for 1 hr. Heat inactivated 65°C for 15'.
5µL RNase-treated fragment
3µL PTZ19mmcII CIP (20ng)
2µL 10x ligase
5µL ligase
5µL H_2O
RT, O/N.

5/18/93 into DH5α 5/19 No wh colon

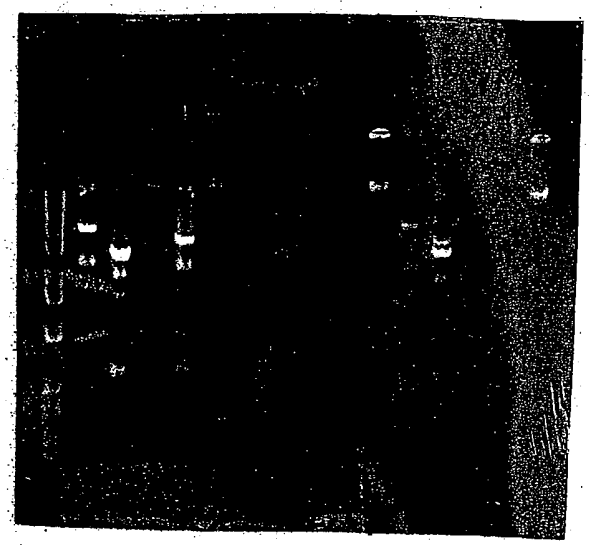
5/16/93
Rest of sample resuspended in 17µL H_2O, 2µL react 5, 1µL RE II. 1½ hrs. @ 37°C.
Adjusted to high salt (2.5µL Mg salt, 1µL NCO, 1.5µL H_2O). 1½ hrs @ 37°C.
Same treatment ± 272-3.

Ran on gel

go to pg 49

Helper d1 construction

8/12 Resuspend pellets in 3 µL H_2O & added 17 µL cocktail (hi salt + Xba). 1½ hr @ 37°C.
- Also set up separate digest of Xba alone. as a check/ control.



Vector site and site in oligo

Only clone having a 600bp fragment in Xba/Rst double digest sample #2. However, ~600 bp fragment in Xba digest alone ??
Maxi Prep of #2. (glycerol stock #14) 8/14/93
8/13/93 Isolated DNA from maxi. PEG ppt @ 4°C for in 3hrs.
quantitated
Rst digest

37° O/N.

- H_2O
- 2 µL DNA

& separate I & helper d1, & 272-3.

8/14/ + 1 mL EtOH −70°C for 30'
d. Rinsed Dried,
Xba digest
resuspended in 17 µL H_2O, 2 µL 10x high Salt, 1 µL Xba 37°C 1½ hrs.
Run on gel isolate
600 bp fragment from helper d1 & very faint
~12.5 kb from 272-3
(No blunt end because compatible ends for ligation)
ligate electrolluted DNAs O/N.
Resuspend
Resuspend
5 µL
5 µL
2 µL
5 µL
3 µL
lig

µL (use 5 µL) (all)

go to pg 1K

Helper BstEII construction

8/17 ligation: 16μl H2O
2μl 10x ligase
2μl DNA ligase
O/N @ RT

8/13/93 Tformation of JM105s c helper BstEII ligation.
LB/AMP 37°C O/N.
8/14/93 No colonies
8/16/93 Repeating 272-3 digest c BstEII & ApaI
religate & tformation
5μl 272-3 (#55)
2μl react 2
1 μl BstEII
12 μl H2O
60°C
cut c ApaI @ 30°C 1½ hrs.

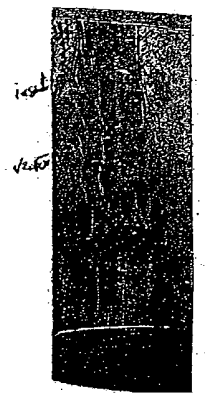
Blunt end T4, run on gel.

8/17/93 Cut out ~7Kb band, electroluted, EtOH.
Resuspended in 16μl H2O
2μl 10x ligase
2 μl DNA ligase
O/N RT

8/18/93 Tformed into DH5αs.

8/19/93 Picked 12 colonies for mini-preps.
O/N shaker

8/23/93 Mini preps cut c EcoRI-HindIII since ma
re-cut #1 & 4
All po
Set
#1
fro
(v



8/25/93

Helper d1 construction

8/15/93 Tformation of DH5αs ⊂ 272-3/Helper d1 ligation
37°C O/N.
8/16/93 No colonies.

8/16/93 Tformed again into DH5αs
No colonies.

[redacted] d1
Re-cut Helper d1 Maxi DNA (8/13/93 p 46)
⊂ RSR + Xba:

6.46μL DNA (5μg)
2.0 μL #5 react 1½ hr. 37°C
1.0 μL RSR
10.54μL H2O
adjusted to high salt
2.5 μL high 10x
1.5μL H2O 37°C 1½ hrs
1 μL Xba
ran on gel, electroeluted, EtOH ppt O/N

8/19/93 Centrifuged, rinsed, dried
resuspended in 5μL
added 2μL 272-3 cut ⊂ Xba/Rsr (prepared 8/13 p 46)
5μL DNA ligase
2 μL 10x ligase
6 μL H2O
16°C, overweekend.

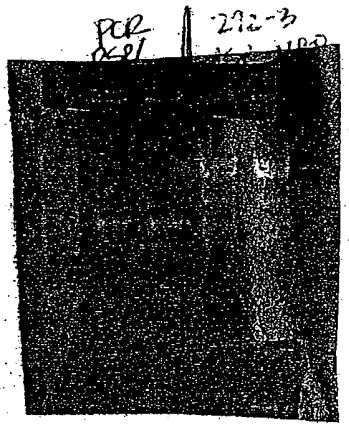
8/24/93 Discarded everything--Not what we want!

Repeat of d1 PCR (as on pg 36)

60.5μL H2O
10 μL 10x (DeepVent)
.5 μL BSA
3 μL dNTP (5mM)
10 μL 2A template (pg 36)
10 μL Helper 3 (G-3) (final [] = 1 μM
5μL RSR (11-4) also
1μL DeepVent Ex0+
File 52 (94-1', 52-1', 72-1')

PCR RSRB- V26Nco
RSR 2 construction

8/16/93



Arrows indicate bands extracted
Electroeluted, EtOH o/N

8/17/93 Centrifuged, rinsed, dried.
Resuspended PCR (Rsr-Nco) in 5μL
Resuspended 272-3 Rsr-Nco in 10μL

Ligation
5μL PCR RSR-Nco
3μL 272-3 Rsr-Nco
2μL 10x ligase
5μL DNA ligase
5μL H_2O
O/N RT

8/18/93 T.form in DH5αs. No colonies.

8/24/93 Took sample #3 (from Deep vent PCR of RsrB-V26Nco (8/15/93 pg 45) removed oil on parafilm adjusted to 0.1M EDTA (~80μL → 1.6mL 500mM EDTA) ran on gel electroeluted. EtOH, -70°, 30'. Froze pellets -70°C o/N.

8/25/93 Resuspended a small sample of PCR product into 10μL H_2O to ligate into PTZ19 hinc II. (a 10μL of PCR sample - ran separate lane of gel)
5μL DNA
3μL PTZ19 hinc II
2μL 10x ligase
1μL (high) ligase
9μL H_2O

8/26/93 T.formed into DH5αs
8/27/93 No white colonies

To rest of electroeluted sample cut Z Rsr & Nco.

go to 52

Helper d1 Construction

8/0/93 [redacted] Ran 10 µL on gel

Removed all ox. solution. Added 1 µL 500mM EDTA (stop Final 10mM)
Ran on gel. Electroluted 1 ton ppt.
1 mg pellets ON -70C.

8/23/93 Resuspended pellets in 15µL H2O
2 µL 10x PNK, 2µL 10mM ATP, 1µL PNK
37°C 1 hr. Heat inactivated @ 65°C 10 min
Ligation to pTZ19 Hinc II:
5µL d1 fragment
3 µL PTZ19 Hinc II (10ng)
2 µL 10x ligase
1 µL High T ligase
9 µL H2O
RT 1 hr

8/26/93 1 form Duss.
8/27/93 No white colonies

9-5-93
4 tubes of "d1 3rd" ? combined → Tag v.s Vent ? ?
Phenol extract
T4 Blunt end
23 µL H2O, 3M 2.7µL
3µL 10x T4 pol
3µL 2.5mM dNTP
1µL T4 pol
37C 20'

Gel
Electroelute
Etoh
1/3 vol TE + 5µL 2.5M NaOAc, + Etoh
-70C 15'
Pellet Dry, Resus
7.5µL H2O
3µL 10x PCR
1µL PCR
Remainder of frag

5µL to mini gel
Ligation
PTZ 19 Hinc 2
Vent 4
Dest 6
16x 13
20 µL
Ligase 2

Pur5 — from p-y 52

Helper 2 construction

- Helper 3A virgo plasmid extracted
- Spin column
- Thermo Wizard column kit ✓
  - 5x2 5 min elute
  - 105 µl 70°C pre-heated ✓
  - Eluted DI
  - 37°C, 20'
  - Alkaline extraction
  - Ethanol ppt
  - Gel fractionate

Continue Pivoting & Labelling Final virgo,
∴ Prepare fragment using V265dco as
template, primers,
Deep Vent Polymerase

	Helper 1 V265dco				Helper 2 V265dco			
	Cocktail							
	5.3µl H_2O				5.4µl			
	10µl 10x				10µl			
	0.5µl BSA				0.5µl			
	3µl 10mM dNTP				3µl			
	2.8µl Helper 1				2.2µl Helper 2A			
	2.2µl V265dco1				2.2µl V265dco			
	1µl deep vent.				1µl			
	1	2	3	4	1	2	3	4
H_2O	16	8	4	0	16.2	8.2	4.2	0.2
50mM $MgSO_4$	0	8	12	16	0	8	12	16
Template 2A3 1:100	10	10	10	10	10	10	10	10

1:100 dilution of Helper #3

to page 55

RSR2 Construction

8/26/93 Cut 2 RSR recognized pellicular [illegible] out
recuts, 1 ea RSR [illegible] ppt
Resuspended pellet in [illegible]
1 ml NaCl, 1/2 hr 37°C
Run on gel. Electroelute. Store ppt.
Resuspended pellet in 100 µl H2O (stored remaining @ -20°C)
5 µl RSR BglII/NcoI RSR-NcoI cut
2 µl 10x [illegible]
3 µl [illegible]
1 µl Ligase (400,000 u/ml)
9 µl H2O
O/N RT
8/26 Transformed into DH5αS 8/27/93 No white colonies)

8/26/93 Sequencing using Kasper reverse as primer
Per Mandel 1/4 prep
Spun Per
14 µl 2M NaOH
37°C 1 hr 20'
2 µl 3M NaOAc
49 µl H2O
200 µl EtOH
-70°C
Resuspended pellet in 7 µl H2O, 2 µl Seq reaction
1 µl Kasper reverse
Rxn'd Seq Rxns per protocol.

8/27/93 No readable sequence

9-2-93
Phenol extract
EtOH
TH Blunt end
Phenol
EtOH w/ NaOAc
TH Ligase
2 µl 4.0 w/ 3 µl 10x 12°C
3 µl 10mM ATP

Helper BstEII construction

8/26/93 Cut ☐ Sal-Nco & Sal-Cvn for confirmation
Sal-Nco gives 1450 bp frag.
Sal-Cvn " 289 bp frag.

Sequencing using Helper reverse as primer.
BstEII Maxi 366ug/mL

32 µL BstEII
8 µL 2M NaOH
37°C for 20'
12 µL 3M NaOAc
28 µL H2O
500 µL EtOH
-70°C.

Resuspended pellet in 7µL H2O, 2µL seq rxn mix, 1µL helper rev.
65°C 10', RT 10'
rest seq. rxns as protocol.

8/27/93 No readable seq

Hinc 2 cont'd.

Hinc 3

1 µl 3 a H2O
10 µl 10x
0.5 µl BSA
3 µl enzyme
Rest Hinc 2 - any size left
2 µl restrict
1 µl 2 cap plant
1 µl 50mM MgSO4
1 µl template pZA #1 / 100

Ran 3 hrs, appeared best at most conc. level

- Heat shocked & iced
- Cell removed via centrifuge
- Rinsed
- Spun down
  - 10 µl 10mM ATP
  - 1 µl 10x T4 PNK
  - 1 µl T4 PNK - 10x
  - 3 µl H2O

Incubate 37° 1 hour

Very low yield. Ligate all to 20 ng pTZ19-Hinc 2 & to DH5α

1 µl elute
3 µl 10x
3 µl restr
8 µl H2O
2 µl ligase

→ No colonies Discarded

Redo Hinc 3 PCR

46.3
1 µl 10x
0.5 µl BSA
3 µl enzyme
12 µl Hinc 3
2.2 µl V26S/C60
1 µl template

	1	2	3	4	
H2O	16	8	4	0	
50mM MgSO4	0	8	12	16	Tubes.
Template	10	10	10	10	

Go to p52

Helper2 (cont)

8/30/93 DeepVent
-template helper 1-NCo. Lfr. 8/26

54.9μL H_2O
10.0μL 10x
0.5μL BSA
3.0μL dNTP
2.2μL helper 2A
2.2μL 26NCo
1μL DeepVent

94°-1'
52°-1'
72°-2'

tubes	1	2	3	4	5	6	7	8
H_2O	16.2	8.2	4.2	0	16.2	8.2	4.2	0
$MgSO_4$	0	8	12	16	0	8	12	16
template #1	0μL	10μL	10μL	10μL	10μL	10	10	10

1:100 dil #3 (8/26)

1:100 dil #1 (8/26)

9/31 Chose #6 to continue Helper 3-26NCo

44.8μL H_2O
10μL 10x
.5μL BSA — new BSA.
12.5μL Help 3
2.2μL 26NCo
1μL DeepVent

Tubes	1	2	3	4
H_2O	16	8	4	0
$MgSO_4$	0	8	12	16
template	10	10	10	10

1:100 dil #6 above

goto 57

Helper 2 (cont)

No evidence of synthesis.

8/31/93 Repeated above also setting up control of first rxn ε 2A as did to obtain #6 product (p 56). Add ~ diluted template 1:1000.

9/1/93 Only synthesis in control sample.

Making Helper 2B to overlap more with 3 (2A → 2B → 3)

PCR 2B

Add to 48 µl H2O
10.0 µl 10x
0.5 µl BSA
3 µl dNTP
82 µl Helper 2B (Final 1 µM)
2.2 µl V26New
1 µl DV

Samples:	1	2	3	4
H2O	16	8	4	0
MV	0	8	12	16
Template	10	10	10	10
1:100 #6-2A				

File 57 (94°-1', 52°-1', 72°-2')
25 cycles, 12° soak.

No evidence of synthesis.

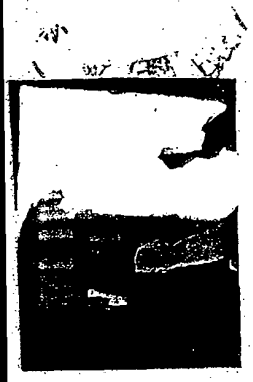
Repeated above ε #6 1:1000, #2 1:100, #2 1:1000 ε control (2A synthesis).
Had to make 10x Deployment Buffer - recipe Promega catalog.

Samples		
1-4	#6	1:1000
5-8	#2	1:100
9-12	#2	1:1000

9/2/93 No evidence of synthesis ✗ in control lane.

go to 58

9/2/93 To 2A-#6 removed oil on Parafilm
set up 3 separate digests (3μl 2A-6 sample)
Ace I 8360
Sty I 7816, 8149, 8226
Dde I 7833, 8456



cut 1½ hr, 37°C. Ran on gel. Appears to be the correct cuts.

9/3/93 Made new Helper 3.

Repeated 2B PCR. No synthesis.
(annealing temps of 50°, 45°, 46°)

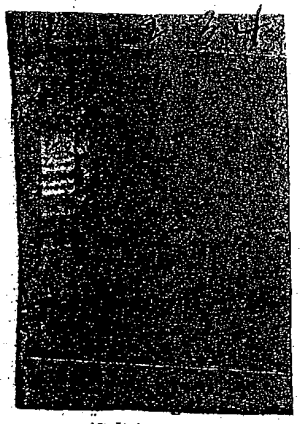
Set up PCR & Helper 3 using 2A product 1:100

56.2μl H_2O
10.0μl 10x
0.5μl BSA
30μl dNTP
1.1μl Helper 3
2.2μl V26NCO
1μl DV

	1	2	3	4
H_2O	16	8	4	0
$MgSO_4$	0	8	12	16
template	10	10	10	10

Helper 3

9/4/93



30 cycles 94°-1', 52°-1', 72°-2'

9/4/93 Appears 1 & 2 non-specific priming
expected a piece ~1300 bp
Set up PCR using 2A 1:20 &
.5 μl BSA & rev.

9/5/93 12.5μl of synthesis

9-5-93

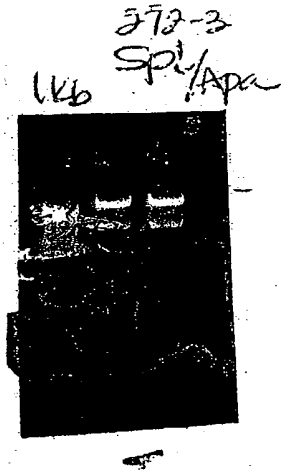
Repeat synthesis of 3 Helper Reverse instead of Gen. — spun down and electroeluted in processing

Use Purified product from slot 80 as template.

Start on page 22 Restriction map on page 152

Go to page 63

Helper Spl construction



9/1/93 Blunt end 20µL DNA (Apa cut)
3µL dNTP
3µL 10x T4 (P)
1µL T4 (P)
3µL H2O
37°C 15', 1µL EDTA
run on gel. Electroluted ~10Kb band
(marker lane ran a little ahead of sample lanes)
EtOH O/N

9/2/93 MP tformed into JM105s. No colonies
↳ 6 came up — Picked

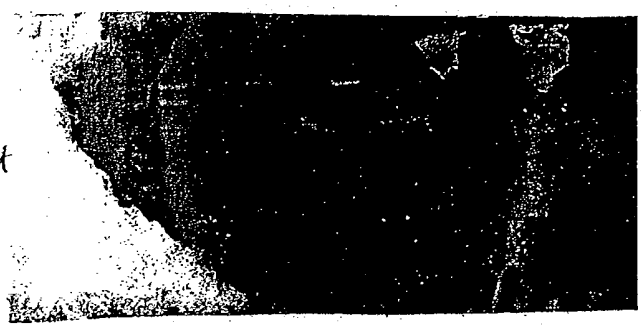
9/4/93 Tform ligation into DH5αs.

9-5 6 105 colonies mini prepd.
Digested with Z1/H3

Predicted Z1/H3 digest should yield fragments of
5'- 2136 - 271 - 1337 - 22 - 4100 - 3'
cut
2136 - 1337 271 3130 - 22 - 188 - 206 - 4100.

All show expected patterns as compared to 272-3 in lane 2

#2 frozen as Helper Spl1 slot 14, Replicon Box



PSR 2 - Nco 4 construction

After the Break
Std 70°C 15'

- Recorded temperature yield
- Harvest with germination into 20 ml

Logic
p72.15 Hive 2

Remarks of Jig with Jig #
Factor 4 (Celsius) Incl. Std
Count 5
10x 3
H2O 16
Lig 2

Turbo Culture
Only background level
Held at 28°
Read 3 for min temp
9-18-93

Temperature Tur
Turbine
3ml 10x
3ml Bam ADP
22ml H2O
2ml PBK
39% Lig
ACE
Jig Check Water with Hive

Of 5 min cycle all cut with Nco.
Boost 4x for virus and digest with 25 Nco, Lig/Nco
Program ACE Box Slot 24
at Lig-Nco
Slot 79

Logistic rule p72.15, Hive 2
Factor 4
Count 5
10x 3
H2O 16
Lig 2
Standard Logic

Does it contain some agarose?
Also include a second ligation with no ADP in ligation buffer
Done 4hr after the PBK
Non-sticky
w/ Std buffer = #2
w/o ADP #2

Rec

9-12-93

Mapi-6 digested with Rsr, Nco1, Rsr/Nco
Results are as expected

Preparation of fragment for insertion into Helper
- EcoR digestion 9/10 with Rsr 2
- Add 6 µl 1M NaCl and 20 Units Nco
- Elute ~1500 bp fragment.

Stored in slot 57
Suspended in ~~20~~ 20 ml H_2O

Preparation of Helper Rsr 2
~~10 ug~~ - 8 µl 272-3 prep
3 µl 10x React 5
3 µl 10mM DTT
15 µl H_2O
15 µl Rsr 2

37°C, 2hrs
Add 3 µl 1M NaCl
15 Units Nco1

Stored in slot 61 discarded

Predicted products
1–516
517–1844
1845–2934
2935–6844
6845–8895
8896–11442

Harvest ~5Kb
1–516 ... 8896–11442
by electroelution into 20µl TE

Ligation

Sample	Vector	Insert	10x	H_2O	Lig
+	5	5	2	7	2
-	5	0	2	12	2

to prep 23

Helper 2 construction - Jaren Lyon-Long

Step 1

Buffer 272-A39 - 100 µl ✓
10 µl ✓
1 µl ✓

Control
H2O 61.4
10x 10
dNTP 8

Primers
22 Help 1 2.7
23 Help Rev 1.4
T4 0.5
MgCl2 4
25mM 6
DNA 10 pL 6, 1, 2, 3 ✓
All three give help product yield
Fig 55

Step 2 - wt Hg titration

	4a	5a	6a	7a	8a
H2O 63					
+H2O		10	6	4	2
10x 10		8			
dNTP					
10mM 3					
Primers (1µM)					
Help 2A 2.1					
4µM					
Help Rev 1.4					
7µM					
Taq 0.5					
MgCl2					
25mM		0	4	6	8	10
DNA		10	10	10	10	10

* 2 pulled 100x

Fig 55

6a, 7a, 8a → high yield

to page 64

64

Helper 2 Cont'd.

5 Lp 3

		9a	10a	11a
Hyo	64			
Hyo		d	2	2
Hos	10			
Dorf	3			

Runners

Hyp 3	1.4
Hyp Rn	1.4
Tag	05
Hy Cl	
DA	

Helper worked 10 hrs
File 55 No Spotters

Repeat Using Helper 28

Repeat Using 28

		9b	10b	11b
Hyo	64			
Hos	10	+ 4	+ 2	
Dorf	3			

Runners

54	Hyp 28	1
	Hyp 4	
A3	Hyp Rn	1.4
	Tag	05
	Hy Cl	4 6 8
	DA	10 c

File 55 9 JB
Ok

Exhibited 1:00 All three worked both

to Page 65

98-99

from pag 24

Legge e continued

9/1/99 Volume of Sphere

12a 13a 14a 15a

4 2 0 1

6 4 2 0

10 10

2 3

Primes 11

23 3

13 12 1/4

2 0.5

4 2

8 4 2 1

10 10 10 10

1.000 100

44.55 No Zeros in Answer

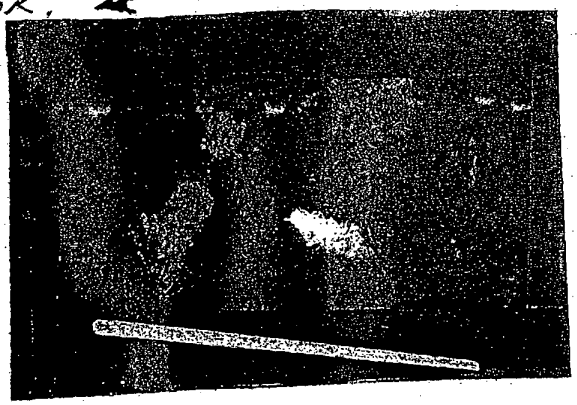
from page 50

d1-Rsr2 preparation

20 colonies picked, ⅓ mini-prep'd.
Digested with Xba/HindIII

Of original 20, 8 contained 650bp fragment.

Digest #16, 20 with
① Xba-1
② Rsr 2
to map & determine if
Rsr2 site is OK.



Xb
Colonies
1—20
X/H3

Also prepare additional ~~culture~~ of 16 & 20 for sequencing.

Xba/Rsr2 double digest failed to release ~~an~~ fragment — Don't know what this means.

q1293 Sequence of 16 very faint but valid
Strand #16.
Slot 12 is replication bug
as d1-Rsr
frag in Hin 2 site of pS7-19R.

Sequencing shows that A→T mutation is in place.

As indicated above, Xba site at 5' end may be missing
∴ 3a all of leader added
Do PCR to see if Helper 2 sequence is present
Using mate 74 deleted 100 bp

(over)

Working Model

Dec 4 [illegible] Helper Mar 94 Form 43

[illegible] 1 2 3 4

[illegible] 10.8

[illegible] 3.3 4.4

[illegible] 10

[illegible] 3

[illegible] 3.6

[illegible] 2.0

[illegible] 3.2

[illegible] 4.4

[illegible]

[illegible]

[illegible] 10

No system except simple P ? [illegible]

[illegible] out his pointers with the [illegible] feature but size [illegible] [illegible] practically nothing.

Rec'd 9-12-93 Using map 94

[illegible] Regt

[illegible] Regt

Long unit for RR	1	2 3 4
30.38 el. HO	0	4 6 10
[illegible]		4.0
[illegible]	10	6 4 0

No [illegible]

[illegible] Map 94 [illegible] 1-10000

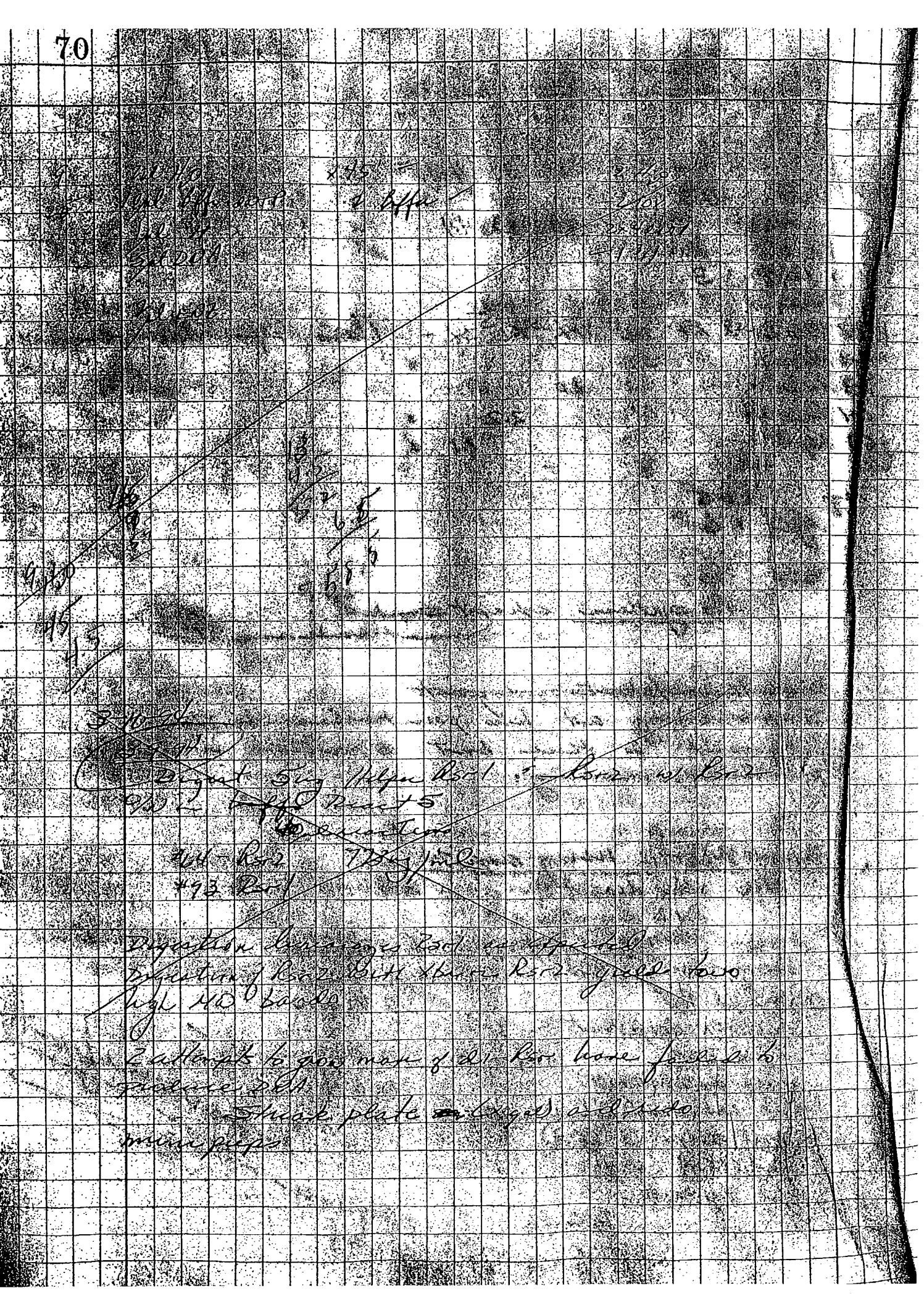
[illegible] Regt 3 (63)

[illegible] (63)

[illegible] Regt

[illegible]

New map [illegible] 3-10-94.



Patient Name: [illegible]

272-3	0.2 ng/ml	85		3	
[illegible]	1.8 ng/ml	83		8	
[illegible]	3.8 ng/ml	82		3	
[illegible]	3.2 ng/ml	48		1	
[illegible]	7.5 ng/ml	24		4	

[illegible] 10 ng [illegible] [illegible] [illegible]

[illegible]

[illegible] [illegible] [illegible] [illegible]

272-3	16	5	16.5	2.5	
[illegible]	30	5	12.5	2.5	29 [illegible]
[illegible]	20	7.5	5	2.5	
25K-3	20	7.5	12.5	2.5	
24-2	30	5	12.5	2.5	

[illegible]

[illegible]

(Notes)

After [illegible] [illegible]

Recommended [illegible]

Estimate to [illegible]

#35	272-3	55.9 ng/ml		12.0	
#41	[illegible]	74 ng/ml		13.2	
#43	[illegible]	94 ng/ml		15.2	40
#47	[illegible]	148 ng/ml		[illegible]	
#49	[illegible]	94.3 ng/ml		20.8	
[illegible]	[illegible]	28.6 ng/ml		8.0	
[illegible]	[illegible]	150 ng/ml			

[illegible] [illegible] [illegible] [illegible]

Agrees [illegible] [illegible], [illegible] [illegible] [illegible]

[illegible]

Discontinue [illegible]

Discuss [illegible] [illegible] appropriate to [illegible] [illegible]

(1) 35 272-3 [illegible]

(2) 41 25K-3 [illegible] [illegible] [illegible]

(3) 43 [illegible] [illegible] [illegible]

(4) [illegible] [illegible]

Discuss [illegible] DOA - [illegible]

Dawn, RN

2

At the end of the reaction
Take a 10 µl sample & transfer
for TLC separation
No organic layer was observed
[illegible] 0.75
[illegible] 0.32
Extract with ethyl acetate
Repeat twice
Combine organic layer
[illegible] 0.46
[illegible] 0.89
Evaporate with rotavap
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
DCM dissolve
[illegible]
[illegible]
To dried
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]

from 62

PREPARATION OF Helper Rsr 2

#45 ~~#21~~, w°c,

~~Rsr~~ fragment still needs sequencing

Initial transformation into JM105 only gave 3 colonies

Miniprep ÷ digest with Xba ÷ Nco I

Expected to yield ~2Kb Xba–Nco + ~5Kb vector + 3' portion of 16S.

#2 ÷ 3 gave similar expected frags ∴ Also cut with Rsr 2 ÷ Nco

Set 40µl reaction o/n at 37°C

After 1 hour, remove 20µl

Add 7µl 4M NaCl + Nco.

Run gel with purified fragments as markers.

Boost #2 for maxi

~~and freeze in slot 27 in~~ ~~Rsr~~ Box

Replica

See page 135

① Kb Helper Rsr 2
② Rsr 2
③ Rsr 2/Nco
④ Purified frag
⑤ 272-3 Rsr/Nco frag

When digesting maxi prep #~~44~~93 (Rsr 1) with either Rsr 2 or Xba 1, a doublet is seen – Why? See page 70 for AGE.

Preparation of dl-Rsr ÷ dl-Rsr 2

Fragment prepared as method for ~~Rsr~~ Eth'rol precipitated and dried as before

Helper Rsr 1 ÷ Rsr 2 digested individually with Rsr 2

Helper Rsr 1 gives a doublet with either – Why? – doesn't know for certain Rsr 1 – demanded maxi

Digest 10µg Helper Rsr 2 with Rsr 2 – O/D.

Kb
Rsr 2 / Rsr 1
Rsr 2
Rsr 1

16µl Rsr 2 ✓
5µl 10×S
28µl H_2O ✓

Cut is complete 3-8-94

Add 5µl 1M NaCl – Complete

Rsr 2 preparation

Helper 2 digested w/ Xba + Rsr 2 complete

1 hr @ 37°C

Add Rsr 1 mg/ml

20 µl ... Rsr 2 digest

5 µl 10x React 5 ... 60 µl

22 µl H_2O

3 µl Rsr 2

4.15 - 0K

5 µl 1M NaCl

2 µl Xba 1

5.05 a.m.

No evidence that Rsr 2 cuts the Xba digestion discontinuous in fragment Prepare fragment from Gel 1 Rsr 2 digestion

3-16-87

1 Rsr 2 digestion

5 µl H_2O

5 µl 10x React 5

3 µl Rsr 2

5 µl 1M NaCl 8:40 p.m

2 µl Xba 1

Resolve and purify w/ Geneclean

Store in set 20 @ 20°C

Purify Xba-Rsr 2 digested helper 2 by ACE - electroelution

Should insert 59

Xba - Rsr 2 digested helper 2

2 µl Hind III plasmid

2 µl 10x Ligase

14 µl H_2O

1 µl ligase

2 to 3 hrs 1

3-17

Biolomics picked for minipreps

Nigatin

8 p.m. 12 -

RVF Religation Construction from pg. 17

Start with
1) Digest mapping 5 RVF segment in pUC Start 11-7-7
2) Re-Purify fragment from 7-7
Equil. DOA ~ 17ug
Sub. 10x High Salt
Equil H2O
Equil RJ
Equil Sal

Mapping on 7-7
1) Pst I ~ 2500 bp insert
2) Xba I ~ 3600
3) Hind III 1100, 1900 700
All as expected

15ul RVF added
→ 3kb fragment appeared as a smear ~ 1/2 of overloading
Purified by Gene Clean in South. 0
→ 5ul/50 to 0% Agarose gel
fragments as seen by AGE

Ligation
92ug/ml

	Vector	Insert	10x	H2O	Lig	
Vector	3	0	3	22	2	~ 10
RVF	2	5	3	17	2	~ 100
Insert	0 ✓	5 ✓	3 ✓	20 ✓	2 ✓	~ 50

Rm T - 90.
2 hours

Pvu II 12 colonies for Ch 1 Cuts. Insert purification
Both vector only slightly contaminated even though AGE [illegible] detect any intact vector.
Conclusions:
Because of repeated failure to clone the fragment - last clone into RJ 5 1/2 segment pTZ 19

Single	Vector	Insert	10x	H2O	Lig	
Vector	2.5	0	3	23.5	1	
Fragment	2.5	5	3	20.5	1	

Numerous white colonies appeared. The gene cassette did confer the intended antibiotic resistance.

Plasmid Work up

2 ml Cs2 15 hr CSSD2 → 50.0 ± Rec'd work

300 ml Ace T

Attempt to determine terminal sequence of CSD2.

11-21

Acc cut release ~ 700 bp frag

Acc Sal dbl

Sal Xmas

30°C 5%

Sal 5hr

Boil 10 min

6 ml 2% HMA Ac

HMA Sepharose → 70°C 15

EtOH

EcoRI Linker ligation

24.5 µl H2O ✓

Rope 100 µM EcoRI linker ✓

Sal concentrated ligase = 2 M U/ml

Sal 10x ligase

Room T 3 hrs 11:30 a.m. to 3:00 p.m.

65°C 15 — Sal to 2 ml 0.1 Sally Ligated

6 ml 10x HYL

Zap 40

Sal EcoRI

Human DNA fragment 5.0762 Digested 15.97

Total Human fragments

Ligate to EcoRI digested Sb-HC 2

Control Pellet

2 Sal Acc ✓

0 Sal Sac I ✓

5 Sal 10x ✓

24 Sal H2O ✓

1 Sal ligase ✓ 5.3 µ Lab 3411 05

11-20-82 Control — no colonies Human DNA digested with Acc I

Experimental ~30 colonies Transferred Determine frag

Digest minis with Nonedetected ← purify by Sal digestion

By By AGE

All measurements are in inches unless
Check fragment for size
(15 & 1500 cut center
Also see Rec 1 cut
Replant - draw diameter in new piece

11-28-93
Groove packed Rl digital → Hogan Replicon Rx
#11 gave a slightly bent Slot 30
Robot by Rx2 - 5th cut. - improving
Rec Hybrid Gene Combo multiple Cut Sites
Blunted fragment end put directly
into Blunted Replicon

Because original fragment apparently
Contains contaminating vector.
Prepare Rl fragment #11 (above) and
blunt end for insertion into replicon

Blunting up Rxn
2 Digest Digest with ClaI in high salt buffer
Use Replicon mini
Use 5x High
Use H2O
Use Klenow Cut
Klenow Repair → Slot 72
DOH-Clean-CP-DOH Slot 8 Frag/mL
Fragment preparation
Clone 11 adjust to high salt conditions
W/ Sal 10x H
9.5 µL
2 µL to R1
KB
H2
R1
R1 Frag in
Slot 78

Klenow
Ace
Clone
Slot 78

to page 29

SSR2 Replica work up — from page 18

Ligation

Sample	Insert	Vector	10x	H2O	Lig	
Vector	—	2.5	3	22.5	2	20
Insert	5	—	3	20	2	20
SSR2	5	2.5	3	17.5	2	room
SSR2 - pET15 #18	✓	✓	5x3 ✓	5x17.5 ✓	5/2	

2 picked from insert plate. What is my vector? Ran BglII digest → Should be 2

10 picks from SSR2 pET15 BglII digested

All are Replica mutn

Redigest plan: Inoculation with DH5α

BglII

Dilute to 250 µl

Buffer

CutSmart

Enzyme

Digest 1 hr

Blunt ended Replica

Sample	Frag	Vector	10x	H2O	Lig	
Frag	5	0	3	20	2	0
#4 Tube 18	5	2.5 ✓	3 ✓	12.5 ✓	2 ✓	~150

Should take these vectors and religate, don't know if it's worth trying

12 picked

Remainder lifted for colony plot

Redo digestion

Gel fragment

Δ Hel 25

BM Kit

Pick 12 + digest w/ BglII

Ecor1/Hind3.

Ran 2 hr

Finger is shot

Only one puncture found

Pst 1 & 2 close colonies

Bgt 2 digest = increases 2 big fragments

Bgt 2 & Clo K vt 2257 in replicas Closing Sol at 2568 and 7800 bp downstream from Acc site used to generate fragment

Vector present in Sterile clone but 2 fragments of 5.9 kb (6?) Prepare two minipreps

Also do Pst 1 digest for [illegible]

Pst cleaved Ryodil 24 vt 2500 bp unrestriction of Acc I site

Pst 1 not predicted to cleave within replication at window 1461 bp. Pst site at 2596 removed by Cla I cut used during preparation of blunt ended vector

Pst digest of replicon yields 2 fragments.

Also

Fragment used was blunt ended Pst frag

Termini are

5' ATCG ... GATT

Vector was fused in Cla I digest

Termini are

5' ATCG ... CGAT 3'

Pst I also cuts replicon at 2136

Partial should regenerate the Pst site

Digest are 3 with Eco RI

Replicon

Smaller fragment on vector

[redacted]

Is it possible that this is original Vector-shuttle?

Pst digest suggests this is so

Do Pst digest of shuttle?

Replicon gives 3 bands

Agrees: 2 comigrating bands

(See page 83)

Ron proj 76

Pat cloning cont'd.

12/5/86 digest of 12/4

Rlact

Blunt 2h 37°C 94 Pel

3 gut H2O 1 gut 20% EtOH

5 ul 10x Stot EtOH

5 ul Sma I dest Dsma 5 U/ml.

Vect 1 Blog Alt - ligate to vect

BTC 20' 60

Stot pptd -20°C - 0/n 1 of gr 131

DTT at ppt'd will be pelleted, washed and applied to gel and extracted prior to further digestion.

Digest with Pst I + Sal

Resolve + elute fragment. - no fragment obvious no DOA obvious.

∴ Known tube 41 5 ul/pl frag in low salt buffer 2h Sequa dest Pst I Known 37°C 20'

Heat inactivate ligate to Thspaind Replicon Cde in #45

	Ligation	Vector	Insert	10x H2O	Lig	Hyg-C	
64	Vector	2	0	3	23	1	?
9	RVF Rep	2	5	3	18	1	lots.
		3x2	5	3x3	3x18	3x1	
		✓		✓	✓	✓	
			6	0	6		
		3x2		3x3	3x17	6	
		✓	✓	✓	✓		

12-15 Plate transformation

Picked colonies onto grid

12-16 Liquid gel pulsing

12-17 Probe synthesis

10 ul RVF frag

Boil 10'

2 ul reaction mix

2 ul dnase - dnase

5 ul 35 - dnase

1 ul Klenow

37°C 30' 65 K/pl

2-2-87 Onto blot 10:30 a.m. negative

Repeat ligation with new fragment prepared by electroelution - Blot 9/

17 Pick clones & transfer - Test probe take good.

Part 1

Re-colonies picked (42)

Set up primary ligation of Frag 2 Rsa-Sac-Hind 2

Prepare frag by Cal digest of 101 Hind 2

electroelute to 2 ml of 101 Replicate

Set up Lig with Rsa-Sac

Rsa-Sac 200 ul 101 Discard Discard

Set up Lig Vector 10x H2O Lig

RVF-Rsa 5 @ 3 10 2 >100

RVF-Rep 5 2 3 10 2 >100

RVT-Rep 5 2 3 10 2

RVF-KS 5 2 3 10 2

Part 2

Transform colonies from Lig preparation

Do the pUC18 transforming control

Hundreds of colonies

Plates saved at 4°C for later picks

Some indication of higher counts on Rep-RVF plate

Part 3

Often colonies were investigated, all six

had negative

Discarded Digest fragment 4101 w/ HindIII to see what it is

Part 4

Many colonies lifted & probed

- all negative

Repeat

16-6 ul frag from 19-7

Repeat vacuum blotted fragment

Sal-Hind digest

Prepare

5 ul NA TA/PL

3 ul Rsa-Sac

3 ul Sac-Hind

10 ul H2O

Ligate 4°C overnight

Discard after 1 week

Part 5

Transform competent cells with Rsa-Sac-Hind ligation 105

Restriction digestion
23.5 + 31.5

Digested 2 ul sample from gel #2
Pool IX
EMS 4x
1 ml Isopropanol

1	11688	
1x	138	
2	10651	bands much too low for
2x	203	ligase work.
3	12564	
3x	733	
4	7010	Discarded
4x	505	
5	11818	
5x	945	

Repetition of transcription

#1 - 272-3 12ul DOA mix

#2 RSt-2 14ul

#3 RSt LSN 4ul

#4 LSt 4ul

#4 Helper BSE 10ul

2ul transcription buffer
10ul RNTP RNTP mix = 2ul each RNTP +
0.25ul 20mM cap
6ul DW + H_2O. 0.75ul H_2O
2ul enzyme mix 1ul 3'-OTP

Incubation

Control — 2ml

272-3 — 2ml

Hepes-Tyl — 2ml

Hydrion 4SN — 4ml

✓ Sue H₂O

✓ Sue 10x Buffer added to each

✓ Sue each with Sue tube cap

✓ Sat 37°C

✓ 10x Spec ← of all w/ G-T

✓ Sue DOA

✓ Mix

✓ Sue Polymixin

60, 37°C

180 Sue H₂O

Sue to each of 2 tubes

DOA 1x

CHCl₃ 1x

Tyl 4 ppt

Control incorporated ~20%

Hydrion Sample ~ 42%

Is the cap analysis the problem

at 500 M seems possible

Is more to alternate need to dig down

DOA panel to use

Cut new DOA pipe with Bunsen

Purpose in concentrated form

20 µg/ml each

Prepare ~ 30 ml H₂O.

DOA

CHCl₃

2-13-73 Tetrad: Not yet dried

$55P2 in replicon

Restriction digest to recombinant 2 confirms that this vector is not the replicon

@ Discrepancy in P3 digest not accounted for by loss of P3 site created by ClaI digest

@ RI digest doesn't account for extra ~2.4 kb band seen in Bgl2 digest unless 2 bands co-run

Designed ~~PCR product to Sequence~~
~~Replicon Insert~~
~~CAG TGT TAA ATC ATT CAG GTA C~~
Replicon Sequence
TGA TGA CAG GAG AAG GGC ATT G

12-11-93
~~Dog~~ 16
1.5ml of 3 day old maxi mag. prep'd
5ml - Bgl2 digest
45ml DW
+11.25ml 2M NaOH - 1PP[10]

12-9-93
ClaI digestion of pUC clone p55P2 released a 5kb fragment. Restricted to release internal ClaI fragments of ~ 900 & 408. Only the smaller is seen, also seen in 55PA-
. Whatever this section is, it seems to contain the expected gene.
. Do manual sequence run!
~~Also what map~~
~~Regina~~
If A is the replicon containing 55P2 it should be possible to map it using Bgl2-SphI double digestion.
Bgl2 cuts at nt 1210, SphI does but cuts just downstream of the insert
. Bgl2 cuts replicon at 2258

If insert is in proper orientation,
Bgl2 → Replicon 5000 + 3000 + 4kb vector.
SphI → linear
Bgl2/SphI 5 kb + 3.3kb + 4kb.

Number 6 consistent the 3/2/72 map
Absorbed 4/27/72

3rd SSM in 60 min Rainy
1396 then

20 cal type 11 W Dell 1/12 Bgz
& fracture direction
Stoned yield + 600 psig bpg
to 7200 psig - 5th magnaling
from 5 KB flag 1 KB 3rd Bgz

Continue mapping A but continue as Blend

2/ SSP2 - is the Z1 fragment in the
Shuttle,
Sinclair the with the original Unocal
promoter constructed, thinned the Apex declines

Mapping SSP-2 & critical first
• Start map & 455P-2
p 55P-2

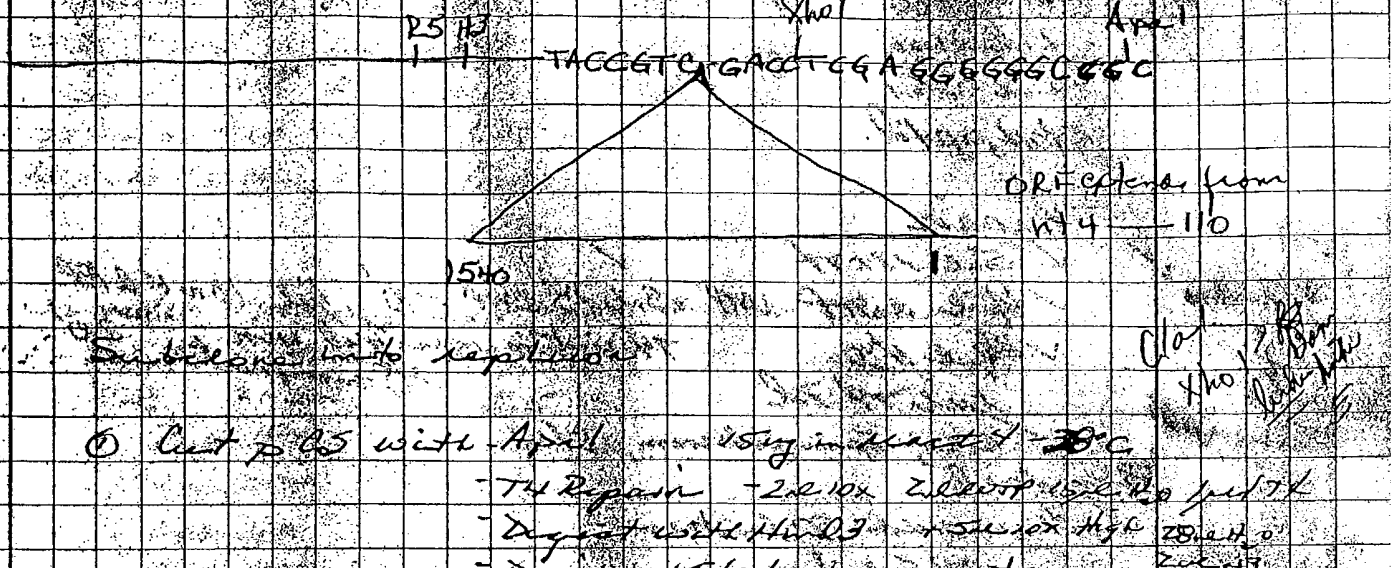
25 ft Need Records —
Digest p-55P-2/Act 1 — Part 1 ✓
✓ Stoned Upper
✓ Heat maturation
→ Fred digest
Unroot longer fragment

Victor Report from
Shuttle 2
→ Need digest ✓
Stoned Upper
Heat maturation
Add Fred
Thrust thrust
Span Columns
CP thrust
Out
Ghost

to page 97

Cloning pKK177 CS gene.

Sequencing reveals that the CS gene is
polycistronic on clone



Subclone this to sequence

Clo I, ? Bam HI
Xho I, Bam HI

① Cut pCS with ApaI 15ug in quant 4 — 37°C
- T4 Repair - 2u 10x 2u H2O 15u 20 per 1X
- Digest with HindIII - 5u 10x Hi-C 78u H2O
- Purify 1540 bp fragment 2u HB

Store in Slot 95

Prepare Shuttle + Bam HI digestion + 15ug Shuttle 1-4
Kleinow repair 2 Ab. Tube 67
All 20 ul add Hind 3 digest Tube 67 gave strange
buffer. Bam HI pattern
Repeat using Shuttle 2
Tube 66

② Ligate and transform

③ Prepare 1550 bp Cla I fragment

④ Insert into Replicon.

Shuttle ligation

	Vector	Insert	10x	H2O	Lig
Vector	1	0	3	24	2
CS	1	5	3	19	2
			✓	✓	
			2:45	12-14-93	repeated

12-16-93 Colonies picked
No patches.
Repeated
12-19-93 3 colonies picked
Cla I digest.
All positive

Restriction of Hind fragments

20 mg - 30 ml
5 ml - 10x Hind
2 ml H2O
2 ml x Hol
2 ml Cla I
Ehol appl'd.

T/Lipase
✓ 5ml 10x TT
✓ 1 ml each - DATP + 3 ml 3 DATP - 10 1 ml 10 mM DATP
✓ 36 ml H2O + Nucleotide monitored with u.v. 20 1
1 ml TT PL 33 nucleotides
Ehol, 2 min column, Ehol
Blunt end
Thermal cycler Protocol
245 ml H2O
3 ml 10x Ligase w/ 1 unit
1.5 ml 10 mM DTT Protocol
1 ml High T Ligase
14°C - 8 hrs

RH - 45°
6 ml 10x Hind
20 ml H2O
4 ml 2nd
37°C 4 hrs

SAE and clutan
Sucked - OK
Shut - slot 107

Sample	Nubs	Sent	10x	H2O	Lig
Vuln	1	0	3	24	2
Cs R1	1	5	3	19	2
	1		3	1	1

Into JM 105 12:21 AM onto X-gal plates

6 colonies picked RH

12:37 PM pipetted KB

R1 cut - R1 cut. KB R1

PGRV

Restriction slot 117 KB CS R1 RH

1 transcription
Neuroplasia - Neuroblastoma

①	[illegible] #55	150 ug/ml	2 ul
②	Helper Rsr #47	260 ug/ml	4 ul
③	Repression #73	7 ug/ml	1.5
④	Helper Spl #94	685 ug/ml	1.6
	HAH #105	850 ug/ml	1.2
	Helper Rsr #23	130 ug/ml	8
	LSW Repression #104	3.2 ug/ml	1
	Helper Rsr #2 64	220 ug/ml	45

Serum transcription - Neuroplasia

20	1 - 2 ul	2 - 4 ul	3 - 1.5 ul	4 - 1.6 ul
40	1 - 5 ul	2 - 3 ul	3 - 5 ul	5.4 ul

8
- 2 ul 10x
- 2 ul ATP
- 2 ul CTP
- 2 ul GTP
- 2 ul UTP
- 1 ul Spl ATP
- 2 ul 0.2 mg

5x Buffer
- 200 mM Tris pH 7.9 — 2 ml 1M
- 30 mM MgCl2 — 0.3 ml 1M
- 50 mM NaCl — 0.35 ml 1M
- 10 mM Spermidine — 1 ml 0.1M
- 6.2 ml H2O

2 ul at end.
4:30 - 5:30

Spl #0 add
2 ul Spl #0

			%	µg RNA	
#1	30	5371	0		
	60	48			
#2	0	5646			EtOH ppt's from 2
	60	514	.062 .092	47.2 ug 15.4	ug in Nanodrop
#3	30	4995			
	60	177	.021	4.49	
#4	0	5536			
	60	233	.033	6.17	Significant

bkgnd = 50

Comparative Transcription levels

	Homemade	vs.	Ambion	Kit
Endpoint	42			
Fwd Endpoint	DNA			

H2O	3	3
Buffer	5	2
MMP	6	8
DNA	2	4
Enz	2	6
RNAsin	2	1
ENZ	2	2
Trans	1	1

Enz in homemade Prep — lot 022312

	Homemade	Yield — Could not get product to elute.
+0	4686	37583
+0 +	169	219
+60	197	1553
+120	300	2517 — Ambion Final product

Homemade +0

Repeat using Ambion Enzyme & prep of homemade buffer?

	Homemade buffer	Ambion buffer	
H2O	3	6	DNA to (buy) kit
5x	4	2	comp.
MMP	6	8	
Buffer	1	0	
ENZ	1	1	
Trans	1	1	Volumes of components in homemade buffer
DNA	2	1	Remake buffer
+0	10688	10936	
+0 +	2241	4188	
+60	869	1656	

to page 93

Cloning continued

Purify Bam HI / Hind III frag from CsCl, R1
└ Slot 9L

Ligate to Bam HI / Hind III cut shuttle 2
└ Prepare from O/N 2ml culture by "magic prep" Slot L6

Ligation	Vector	Insert	10x	H2O	Lig
Vector	1	0	3	25	1
+CS	1	5	3	19	1
	✓		✓	✓	

12-26 Into TH105 a.m.
— Cell colony picked — Prepare tiny prepare
Decided must prepare tiny prepare additional from new master prep.
└ Done — 1.5 days ahead!

Insert ligated into restriction prep.

	Vector	Insert	10x	H2O	Lig	
Vector	2	0	3	24	1	13 colonies
+CS	2	5	3	19	1	>300 colonies
	✓	✓	✓	✓		

Into DH5αF' (12-26)

Picked 8 colonies for Cla I digestion

→ Is this R5+ contamination of fragment?

12-28 Cla I digest.
All are positive
Frags #1 in Slot 31 of Replicon Box
as CS Shuttle

1-20 Start culture of CS Shuttle for cloning with replicon
Prepare fragment for insertion into BL Cut. Cla I
replicon
Frozen in Slot 10D
Digest with Cla I and prepare fragment for insertion into replicon and Bluescript ~1.5Kb

		10x	H_2O	Lig	Count	Lig
Rep CS	2	3	18		5	2
K5 Cu	2	3	23		0	2
K5+CS	2	3	18		5	2
					2	

2 colonies from Rep Cloning
#1 had 1500 bp frag

Digest by RE/Mung Lig
+ incubation 30 min 37°C

No frag seen

Cut inserts w/ Eco RV
Need to verify double digest
OK
Store as Rep-CS ; slot 20 in nylon box
Made stocks O/N

In vitro transcription

from p. 92

1/23-24

New Buffer

5x =	200mM Tris pH 7.5	2 mL 1M
(46)	30mM $MgCl_2$	0.3 mL 1M
	10mM Spermidine	0.1 mL 100mM
(125)	50mM NaCl	0.5 mL 1M
		7.1 mL H_2O

Combine all reagents except spermidine to full volume of 9.0 ml

Autoclave

Add 1 vol % 0.1M to 0.9 vol % 5x-Buffer when ready.

Note that DTT needs to be added when assembling reaction

Assembly of reaction - at room T

20 µL	5x
10 µL	100mM DTT
100 µL	RDA Sin
20 µL	RNTP
	DDA
	H_2O

Mix : spin

µL TT pol

= 100 µL total

1/23-24

Control Reactions

Does this work now -

Control DDA LSN - Dot 1. 289 µg/ml

Reactions				A	B	C
(A)	1 µg DDA	1/60	5x	20	20	20
(B)	2 µg	1/30	100mM DTT	10	10	10
(C)	Noise	1/15	RDA Sin	2.5	2.5	2.5
	samples	1/36	15mM RNTP	12	12	12
Spl per µl			3µl conc	45.5	41.5	49.5
g/c	124/132.5		H_2O	48.5	44.5	52.5
			DDA	4	8	0
	Promega	—	TT Pol	3	3	3
			3µl RNTP	3	3	3

No det incorporation
w/ Rxn go TT, 2% at 0.2 OD 312 nm

Repeat labeling Rxn, TT, Phyaerin

3 reactions
A = 1 ug LSD rep
B = 2 ug LSD rep
C = 0 no DOA

50 ul/rxn

	A	B	C
5x	10	10	10
100 mM DTT	5		5
2% Asin	2		2
3 mM ROP	12		12
3H-rUTP			
DOA	4	8	0
H_2O	12	8	16
TT	2	2	2
3H	3	3	3

5% 60 min, 37C

to	126932	125565	119238
DOA	579	432	797
+	3165	7343	396
6V			
	3.1 ug	10 ug	0

10 ul/rxn

RNA production

	74 ng/ul	96 ng/ul	116 ng/ul
	272-3	Rep-LS	Rep DOA
5x	20		
100 mM DTT	10		
2% Asin	3		
3 mM ROP	20		
3H-rUTP	3		
H_2O	6	6	6
TT	3		
DOA	35	35	25

File samples

2 ul

90 minutes

to page 95

In vitro transcription Exp 1/27-91

	272-3	2p-CS	HA
t_0	1473	15917	14866
t_{Tr}	122	1617	92
t_{20}	87	521	2756
Net	0	354	2664
%		02%	0.17%
nMole/h		31	268
ug DDA		9.9 ug	83 ug DDA.

3.75nM each
375nM each
1.5ml total available.

after phenol and addition of 120Ac, CHP added, solution became very cloudy. Probably due to DMF?

Repeat Digest time 3hrs.

①	272-3	-digest to	1.5ug	20ul	
②	LSN		2ug	8ul	
③	5pl		2ug	15ul	
			1	2	3
3.5	5x	20			
1	100mM DTT	10			
1	5mM NTP	20	4 each		
3	H NTP	3			3 clear tubes
1	H_2O		21	34	26
	TT (RL)	3			
	DDA	1	20ug	8	15
1	RNAsin	3			

12:55 p.m.

No incorporation Again

Second Run RNAsin

Repeat pTV9 Control - See Scan digested pTV.18.

Control transcription

5x zonule

100mM DTT	10
15mM	60
NTP	
Sub cocktail	
3HUTP	3
RDASm	2
H_2O	40
DDA	3
RT	2

Sub sampling at
0, 30, 60, 90, 120, 150, 180

DDA = Sm1 Cocktail pT2.18
OD 260/280 ~ 1.7
339 ng/ml

RDASm Promega 7,93
H_2O Nuclease
No incorporation

Repeat using HAK/Ingels kit
of 3HUA or 3HA
Stock 1:15

Repeated using Table 3-UA + 3H-A

3H-A	3H-U
38570	38799
785	194
444 244	1357

0.029x
= 44.9 μMoles inc
= 15 μg RDA

3.75 mM dNTP
3.75 μM/ml
375 nM cocktail
1.5 μM catalytic

1.99 Repeat again 3% cocktailing

	A	B	C
20x	20		
100mM DTT	10		
15mM NTP	12		
3H-UTP	3		
RDASm	2		
H_2O	13	36	16 ✓
DDA	8	15	35
RT	2		

A = Hall LSI
B = Spin SA
C = Bulk CS

No incorporation again

10 pg

1-29-91 (A)

from page 86

Ligation

Vector = H3/BHint/R1 Shuttle 2

	Vector	5mut	10x	H_2O	Lig
1-30 (1/30/91) Vector	2	0	3	23	2
1-30 Vector + SSP	2	5	3	18	2

1-30 Into JM105

1-30 (Gan)

1-3Φ

No obvious increase in colony # between the samples.

Pick 2 anyway.

All vector

Prepare new vector

- 15ug digested with Hind3 in 50ul high salt
- Add 2.5ul 1M tris pH 8.3
- 2ul CIP
- 42°C 20'
- ΦOH
- EtOH.

Resuspend in 50ul high salt

w/ 2.5ul eNTP

5 Units Klenow

37°C, 30'

ΔH-70°C, 15'

- 20 Units EcoR1

1hr, 37°C.

Continued mapping of pSSP-A.

It appears that SSP-A is SSP in the replicon.

Do control R1 digest, Clal of replicon.

Still don't know orientation

Make frozen culture stock of this clone - JM105

KbWalker

pSSP-A

ClaI

R1 / ClaI

pSSP ClaI

Shuttle 2 H3

Have HindIII cut?

Because ClaI digestion of the SSPA clone yields only the 400 bp fragment a single cut upstream but should allow the determination of orientation.

Num1 cuts SSPA one time
∴ Cut with ClaI ÷ Num1
+ orientation showed yield ~ 1Kb
- orientation showed yield ~ 3Kb frag
in addition to ClaI frag.

A/so ClaI digest of 107 should leave remainder of plasmid intact — is Num1 co-running doublet.

Do R1/Nh3/Cla ÷ 4.3 digests.

Plasmid is as expected

→ negative orientation
3Kb frag released
Discard

However, ClaI digestion suggests that it should be possible to clone ClaI frag from SSP in shuttle w/o the replicon directly
∴ Purpose ClaI frag from pSSP2 from
pg. 76

To pg 103

from 5-yr 77

In vitro transcription

Why consistently no incorporation?
of RDAa?
Dead enzyme?
Bad buffer?

Need to do additional controls
Position control Randy-5' clone?
1- Run control Hg agarose gel - homozygotes ~100mA
2- Set up mock reactions with available internal members.

Set up reaction by standard protocol.

~~10ul 5x~~ ~~20ul 5x~~
~~Gul 10TT~~ ~~20ul 10TT mix~~
~~Balance dH2O~~ ~~5H-WTP~~
~~50ul~~

		①	②
10	5x	20ul	22 10 Two reactions
20	100mM DTT	10ul	10 5 one w/ template
40	10TP	20ul	20 10 pT18 Seal
6	3'-WTP	3	3 1.5 A second with 5'e RDA ladders
4	RDAsin	2	2 1 control w/ 5'e DDA also but right
40	H2O	36	33 H2O 10.5 not monitored
	DDA	5	5 + 5 RDA
	TT Pol.	2	2

Cocktail 2 times

5 loop/lab from #1 only

20 µl removed from #2 set to -70 - frozen at w/ transcription kit.

→ Ack = no indication of breakdown — gel not run that — questionable reac

Control gel

Lane	Samples
1	E6 RDA 5'e
2	BRL Ladder 5'g
3	~~Exchanger ladder~~

2-3-83

Rate of UV transcription

Phenol extracted VV
Isopropanol ppt'd.

Suspend in 10 uL TE 1/10
5 uL + 10 uL Hg Simple Buffer

Lane		
1	RDA beads	
2	pTZ18 Sca I	
3	LSN 2-1-83	Note that none show
4	CS 2-1-83	significant incorporation
5	[illegible]	

70 mA, 1 hr. No bands present as expected

Repeat using BRL Buffer + Sca - pTZ18 template according to BRL protocol

6 uL 5x BRL Pol buffer
1 uL dTTP mix — 3 tubes saved
1.5 uL 100 mM DTT — (3:20, 3:10, 3:30)
3 uL - 1 ug template — 5 hrs, 4 completed
3 uL - ^{3}H-dTTP
14.5 uL H_2O — TCA precipitate 3:30
1 uL BRL T7

t$_0$	41448	
t$_0$ +	369	
t$_{40}$	3485	
t$_{100}$	3454	5 3.75 uL NTP final
	=0.0744	150.8 nM available
		11.19 cpm/cm = 3.5 ng 35
		not great

Nitro transcription

Repeat with reaction w/ 2x as much RNA
DDH$_2$O 2x as much R.O. with 3rd RDA (B)

	A	B
6ul 5x BRL Buffer	✓	✓
2ul 75mM RNTP	✓	✓
1.5ul 100mM DTT	✓	✓
Template	6	3
3ul ^{3}H-UTP	✓	✓
H_2O	10.5	12.5
BRL TT Pol	1	2

3ul t_o, t_o, 60 → 120 t_f = 2:00

RDAsin-1 left out — PTE 18 Sec

RDAsin added at 30 min

within 30' B precipitated while incubat

Both became cloudy during the first hour. ✓

	A	B
t_o -	51949	53699
t_+	77	167
t_1	2046	1475
t_2	653	2414
Net	1469	2247
%	.038	0.042 → 6.15
nM inc	5.69	205 → 6.15
ug RDA	1.723 avg	1.92 ug

Both were very turbid at end of incubation period. Why?

15% 75 nM = 11.25 nM

Why the cloudiness
Set up reactions again in 100µl samples w/:
w/o Pol and incubate at 37°C.
Use one of helper DNA's, 2ul TT Pol. - 45N as template

w/ BRL Buffer
20ul 5x
15µl RNTP
5ul 0.1M DTT
DTT Template

RDAsin-1
B$_{12}$-MOP-3
H$_2$O
TT 2

3ul samples

t_o - 15332
t_o + 31
t_+ 331 - 1.3
t_1 154.3
120
t_+ 60 120

~~11/19~~ 11/20 In vitro transcription

① 272-3 ~~2~~ 1 ug/ul

② Ref 1 200ng/ul

③ BSTE2 ~~39~~ 19ng/ul

20ul 5x
5ul 100mM DTT
15ul H2O
2ul RDAsin
Sig

	1	2	3
5x	20ul		
100mM DTT	5ul		
H2O	20ul		
RDAsin	2ul		
3HA-TF	3		
DOA	20	10	5
H2O	~~33~~ 26	~~43~~ 38	~~48~~ 43
R-7	2	2	

37°C 1hr 3ul sample

7:45

2ul DOAsc 37°C 15'

15ul H2O
15ul CH4OAc

No incorporation

Phenol
CHCl3
2.5 volume Isopropanol

Redo the DOA preparation.

SSP-2 replicon insertion (2-4) from pg 2

Using pSSP-2 as source

- Prepare ClaI fragment ~ 3Kb SSP-102 ~2
- ligate into ClaI-CB replicon

Why does this work

Digestion of pSSP2 - contains Acc I & Acc I & EcoRI frag - with ClaI cuts only at 3765 & 4128 sites.

∴ Cleavage with ClaI cleaves at ClaI site found at 3765 → 3 Kb containing entire ORF

Also ligate to Bluescript for sequencing

Ligation

Sample	Replicon	Bluescript	Buffer	10x	H_2O	Lig
Frag	0	0	5	3	21	2
Replicon+	1		5	3	19	2
Bluescript+		2	5	3	18	2
Rep Vector	1		0	3	24	2
	✓			✓	✓	✓
				4x3	4x16	4x2

~~Very small amount of fragment obtained~~, ~~spotted~~ ~~others~~ magnetic

2-8-94 a.m.

2ul into LE DH5 α Stated 8:00

- frag had extreme vector contamination

Prepare new fragment

2-18-94

Two prep of frag prepared and ligated - Kyle KS

Stored - Slot 102

2-19-94

24 colonies grew from 3ul of ligation #SSP into replicon vector - 2ul of vector + 30ul H2O

Pick all 24

- All negative -

~~Returned~~

2-24

9 colonies picked from SSP2-Bluescript ligation

ClaI cuts → Only 1 clone w/ plasmid + frag insert

Wk 12 Insert #9

Ground for sequence

Stable Cell lines in slot 32

of liquid box

SSP-K5

Do mini-prep

Restrict 2 3:1 cuts of

SSP-K5 + pJ55P to confirm identity

of insert.

→ Fragment seems a little small.

Kb

107 pol Clone of

SSP-2

Pst1

Bgl2

Hin1

SSP-K5

Pst1

Bgl2

Hin1

Cla1

pSSP-2Cla frag

Products as expected

except for missing Bgl2 site in

SSP-K5 and lack of

visible Pst fragment.

Should have visualized small

fragment

∴ Do sequence ?

Southern blot

Gel layout

33-ladder	pJ55P	SSP-Stuffer	SSP-K5	SSP2 Cla frag	Cla2
45 kcpm/μl	21/43	Cla	Cla	2nd	
150 kcpm/blank					

Probe with 33-Cla2 to SSP2 Cla frag as in Lane 5

Blot preparation

100 Volts

- 0.5M NaOH/1.5M NaCl 45 min
- 0.75M Tris pH 7.5/1.5M NaCl 45 min
- Downward blot as for 10x SSC

107

4-22-94

~~Bam~~ cloning cont'd Blunt end.

5 uL EDTA ✓

H2O ✓

0 CH ✓

~~5~~ - column - ~~Column collapsed~~ | prepare new plug ne
6 gm/pl | from new micro prep

EtOH

Digest with 5 and 7 Pst I prior to purification of fragment.

4-12-94

~~Ecor~~ Pst 265 in pTZ back bone

Digest with Pst I

Add 25 uL Sac I

1.5 uL AGE to monitor cutting of frag

~~Confirmed~~ ✓ confirm

~~Ethanol~~ ppt'

~~Phenol~~ c/o Jason

~~Bac~~ 10x buf

~~Bac~~ H2O

~~Pst~~ Pst I

~~Add~~ H2O

~~30~~ 37°C — Phenol

~~Sal~~ digestion — EtOH

Adaptor ligation

~~5 uL~~ 10x ligt

~~5~~ uL H2O

~~3~~ uL ~~Sca~~ I

~~Purify frag, ligated to blunt end Stuffer Hind Site~~

Ligation

23.5 H2O ✓

1 5 uL adaptor ✓

3 uL 10x HEB ✓

2 uL ligt C ligase ✓

14°C O/N

4-23-94 EtOH ppt'd Frag in 20 uL dH2O

Sca I digest — 5 uL 10x 3

AGE — 3 uL H2O — 50 uL vector (autoclave)

PAK — 2 uL Sca I 8:15 probable.

Ligate Electroelute.

(over)

4-24-84

Ligation of EZ65- adaptor to pBluescript

DNA in Zone reaction
ΔH 45°C, 30'

Ligation

	Insert	Vector	VEB 10x	H_2O	Lig
Fragment	3	—	3	22	2
Vector (3)	—	1	3	24	2
E+Vector	3	1	3	21	2

Note - fragment may be contaminated w/ vector ✓ ✓ ✓

Fragment stored in Slot 77
Ethanol stored in vector.
Repeat ligate. w/ into Zgt11 cut vector
Background from fragment is white.
And BB is gre..
-5: Don't further manipulate fragment

4-26-84

New vector	1	#32	10.9 ug/ml	ligation repeated	
Dephosphorylated	1	10x Kpn			
Vector	0	2	2	23	2
2 best colonies picked for	1 ✓	2 ✓	3 ✓	20 ✓	2

Lysis
3ml TE added
2ml 10% OA
1 ml EtOH
Pellet w/ 15K.
Resuspend in 10 ml H_2O

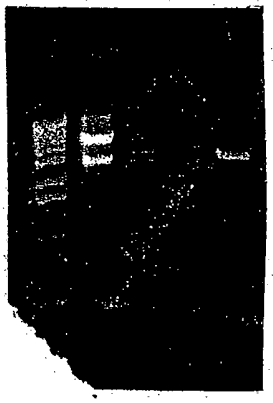
DOA + cells.
Electrocompetent

from prgm 10/
2-23-94

Southern Blotting

Probe Synthesis:
+ 2ul SSP-2 clon frag - have 5 in figure 4/02
7ul H_2O
Boil - 10'
Quench
BM Kit.
3ul dA, G, T mix
2ul Tdn mix
5ul ^{32}P-dCTP
1ul Klenow
37°C 30'
5ul EDTA
45ul H_2O
Spun column
1ml = 177K. cpm. in

2-24-94
HYBRID RESULTS ARE AS EXPECTED.
PREPARE ADDITIONAL CLAI FRAG FOR CLONING.



- legend on Coupe blot
ligation.

SEQUENCE OF DOA in Tube 87 YIELDS UNKNOWN SEQUENCE.
COMPARED TO ALL FILES ON COMPUTER YIELDED NO HOMOLOGY.
SEQUENCE SAVED AS 'MYSTERY'.....
DON'T KNOW HOW TO RECONCILE WITH THE SOUTHERN.

Discard DOA from Tube 87
Discard frozen stock of cells in Slot 32
Redo cloning from original clone

(OVER)

[redacted]

Happened that the [redacted] sent to [redacted] cut

8/30 – not shown – the [redacted] eyes like the insert

– 9/1 Digest 100 µg/µg in [redacted] check 10 in medium sized [redacted]

– 9/2 – almost pupated

– Ligate [redacted] 9/9

(= 3rd ex Ligase)

✓ 10x Ligase, 10µl

✓ 23µl H2O

✓ 65°C 10'

✓ 6µl 10x High

✓ 23µl H2O

Store Cooler

37°C 4hrs 9:15

Harvest by electroelution Slot 18

Fragment [redacted] clean

Ligation to pET-[redacted] Vector 9/7

	Vector	Frag	10x	H2O	Lig	time
Single	2	0	3	23	2	0
Vector Ctrl	2	5	3	18	2	2 – Both pulled
Frag alone	0	5	3	20	2	0

Ligase – 9/10 ✓ ✓ ✓

Digest 5 MIPS

2/25/94

Cosmid transcription - A new beginning

DPOA gels - Methylmethacrylate
1% Ag. Honeycomb
A - Time + Voltage - Investigate
Load & Polarity
Compressed Neo DPA.
V 70
100
H - Gels
Start ethanol increase 61.5 hrs
Expose Neo DPA's on wheat.

Repeated BRL protocol with newly prepared
Seal extract buffer
Modified extraction buffer + 3% incorporation
Phenol BRL buffer
2 Sarkosyl DTT
Pellet Resuspend - Sarkosyl 75mM
125 µl Pellet - Proteinase
2 µl 3% SDS
16.25 µl H2O
5 µl DPA 45 deg/red
1 µl Sarkosyl 77 BRL

3 ml samples
0+
0+
30'
80'
— No incorporation —

10 µl dna + 10 µl Ag Buffer
Each DPA markers.

2/26/94

	C	D
Cosmid Transcription	Control DPA	pTZ18 Sa
77 Buffer 5x	40 µl	40
Equimolar Resp - 25mM Each	6 µl	6
Control DPA	1 µl 2 µl 3 µl	2 2 µl 3 µl
H2O	10 Sarkosyl 0	4 Sarkosyl 0
Enzyme Mix	2.0.	1 µl.

4 - 3:50 4 - 7:30 Ind sect 100

Separated DNAs treated 15' From - Rox DNA

1.5mM	4- 20746				
7.5mM	6- 146				
15mM	7- 3823	3.37	0.19	18.0	32.42
	8-				
	9-				
	10- 24923				
	11- 138				
	12- 5378.	5.40	0.22	18.0	41.09

→ 600nM unstable in 20% Human serum

1 PREC

Repeat Temperature

1- Control pET-21b-Xa
2- ~~872-3~~ H1441
3- LSH
4- RSH-1
5- RSH-2

Repeat digest of pET-272

20% reaction to mount for delete DOA

	1	2	3	4	5
1.5x buffer	6	6	6	6	6
1.25mM ROTP	9	9	9	9	9
H_2O	8	4	6	5	2
1 3H-ROP	2	2	2	2	2
DNA	2	6	4	5	8
T. Pol	3	3	3	3	3

4- 950
14-16

1.5 μg per pcs

DNA treated Jugno 402

Conc	0.48	0.22	0.16	0.17	ng/μl
yield	132	61	45	47	48

Adenovirus transcription cont'd.

272-3 Not 4744 ug/ml
PEG-1 Not 1.2 mg/ml

	272-3	PEG-1	
5x Buffer	6	6	
25mM NTP	9	9	
H_2O	8.5	9	
^{3}H-NTP	2	2	1.5uL samples
DNA	1.5	1	
TTP	3	3	

9.85 — 1.00

272-3 0.17 OD
or 83
÷ 1055
15.7 ug

3-24
Replanned turn of PEG-1 showed very little DNA despite high OD.

Prepare new prep
630 ng/ml - 200 ul
Clean By ABE

Cut 20ug with Dot 1
DOH 4
CHCl$_3$ 1x
Extract DNA.
Suspend in 50uL H_2O
1:50 OD_{260} = 0.66 260/280 = 1.71
= 990 ug/mL.

Transcription of control

	#1	#2	#3	#1 1ug 272
5x	6	6	6	#2 PEG-1
25mM NTP	9	9	9	#3 272 + PEG.
H_2O	8.5	8.5	7	
^{3}H-NTP	2	2	2	
DNA	1.5	1.5	1.5 + 1.5	1.5 uL samples
TTP	3	5	5	t_0 = 2:45
				t_f = 4:45

	#1	#2	#3	
-/-	12113	11525	13545	#1 - 272-3 VGE
+/+	292	244	194	#2 - PEG-1
+/-	1375	307	1006	#3 - PEG-1 + 272-3.
net	1083	63	812	cpm/ug 869 cpm/ug
%	0.089	-	0.059	
nM Inc	80		54	
ug RNA	24.9		16	

Promoter in PEG-1 is not functional inhibition of incorporation is not due to impure DNA

3-5-94

Previous transcription reactions
2, 3, 4, 5 of 2764 and
#1 shown above

200 ul TE
OH 1x
$CaCl_2$ 1x
28 ul 2.5M NaCl
700 ul EtOH
-70°C.

Pellet on TC
Rx
resuspend in 50 ul H_2O - Freeze o/n -70°C.
½ ul
use on agarose gel.

Spec activity available only for #6 869 cpm/ug
extrapolate for others based upon this

		cpm/2 ul	ug/ul	½ life
HAM	2	438	0.25	8 d
LSN	3	649	0.36	5.5
LSN-1	4	833	0.47	4.2
LSN-2	5	371	0.21	9.5
272-3	6	443	0.25	8

all OK by ACE

to page 119

2-16-94

5SPZ cloning continued from page-108

Because of difficulty in cloning into Shuttle -
① redo transformation again ← JM105 - still no DH5
② try cloning into pT219R

Why - will it clone into another vector

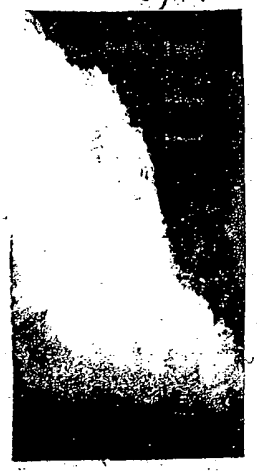
3-1-94

Picked #s from shuttle cloning
#6, 9 look possible by R1 digestion
Digest with Cla I → minigels -

It appears that both are true positives
Boost #6 for maxi to subclone to replace
large 5SPZ-Shuttle clot 22 #9 Shuttle #2
Grow new mini preps of this culture and
that in clot 20 : Compare. Digest the same.
Start maxi of 22 for replica insertion.

Cla I digests 6 & 9 are different.
All the expected fragments are there but
① the mobility of the two large fragments are
different. Taking into account the orientation
of the R1 : Cla-MCS site, doesn't explain the difference.
② It also appears that the long loops are too great.
#9 appears to be closer to the expected size.

6, 9 Cla I



Digest #6, #9 and purified fragment
with Cla I
Also includes previously produced
clone - clot 20 - and cut with Cla I
and Hae III (Hae III included because
first three were ligated with
Hae III before addition of R1
linkers) See page 106

Bands too faint to photo but all
appear to be migrating
Repeat with maxi of #6 - (6+11)

3-9-94

Prepare by Geneclean 3 Kb Cla frag
Ligate to Replicon - Cla
Frag 5 / 10x3 kb
Vector 2 Hor 20 / Lin 1

Robert 3-11-94 (over)

Clones
SSP2-Cla frag 16
SSP2+Replicon 5B → Pooled 12
digest with Cla I
Include replicon as marker

Also digest position with
R1

Cla I digests are negative again

#6 = 9 is possible + digestion only partial

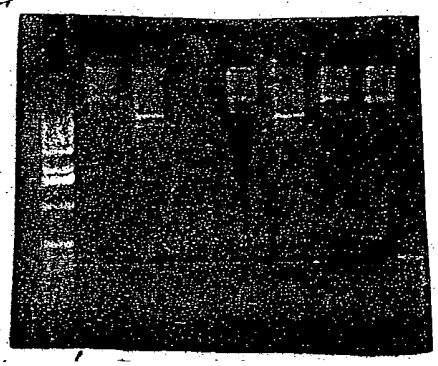
Digest O/N with
Cla I ~3Kb insert
Mun I
Bgl2 - cleaves into 2 remaining pieces

3-B-94
Both do contain the inserts.
Mun I digest releases small fragment.
Mun site in SSP2 - 888
Replicon 7680 - downstream of cloning site
7670 (12 nt)
Small fragment indicates negative orientation
~760bp - Too long?

Do 12 additional mini's
as well as Mun I digestions of
1, 4, 5, 6, 7, 8, 11

Assuming that Cla I site at nt 2874
is the one which downstream Mun I digest
should release fragment of ~2.9Kb for
+ orientation

Only #5 gives an approximation to
this but an additional fragment
is present in 5
∴ Amplify both for
additional mapping.

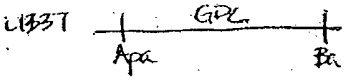


Mun I digests
1, 4, 5, 6, 7, 8, 11

3 additional possibles from
Second batch of 12 are
Mun I digested C, D, J.
D = J + Mun I → 1.6Kb shorter

Subcloning Lassa GPC

3/9/94 Subclone Lassa GPC into replicon shuttle vector II.

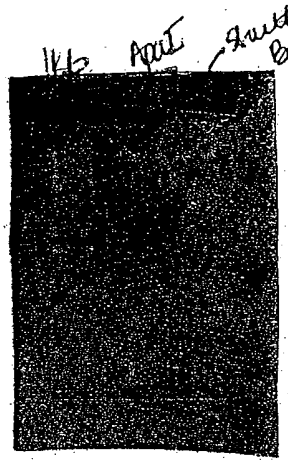


Cut 5µg L1337 (#65 15.3µg/µL) 1µL
react 4 2µL
ApaI 1µL
H_2O 16µL
25°C for 1½ hours

L1337 4100-4700

Cut replicon shuttle vector II (5µg) ē BamHI
5µL shuttle (#70 ; 194µg/mL)
2µL react 3
1µL BamHI
12µL H_2O
37°C for 1½ hours

shuttle 7Kb



added another µL of ApaI to L1337
Cut for another 1½hr.
increased temperature to 30°C let go O/N.

3/10/94 Took both samples Ø extracted 2x ē EtOH ppt.
vector -70°C till ready to use.

L1337 ApaI cut. Centrifuged, rinsed, dried, resusp.
in 23.5µL H_2O for 94 blunt rxn
added 3µL 10x T4
1.5µL 5mM dNTP
2 µL T4 DNA Pol
37°C for 30'

5µL EDTA, 1.5µL H_2O, Ø extracted, spun column
EtOH ppt 30' ; -70°C.

Resuspended pellet in 24.5µL H_2O
3.0µL 10X ligase
1.5µL BamHI linkers
1µL T4 DNA ligase (NEB 400,000

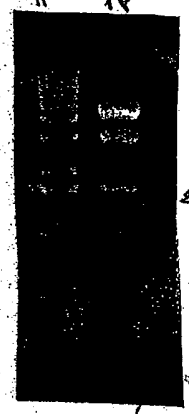
RT ; O/N.

go to 111

Subcloning Lassa GPC

3/4/94 Heat inactivated NZ 70°C for 15'.
60.5 µL H_2O
12 µL High salt 10x
7.5 µL BamHI
37°C for 4 hours

11/4 L3357 Apa-Bam



gene cleaned; ran on gel, expect a 1.5 Kb frag (Bam-Apa-Bam) containing GPC.

← cut out, electroeluted, ethanol ppt -70°C.

3/13/94 Resuspended pellets in 100µL OD_{260} 1:10 dil.
V = 1.03µg i = 200ng

CIP treatment of vector: 5µL 20x CIP
4µL H_2O
1µL CIP
42°C for 30'.
Phenol extracted 1x, chloroform 1x
Ethanol ppt -70°, 30'
Resuspend in 200µL (20ng/µL)
1:10 OD_{260} = 0.0323 16ng/µL

set up ligations (20ng vector)

CI vector alone	CII vector+ligase	I 2V:1i
1.25µL vector shuttle 2	1.25µL vector	1.25µL vector
28.75µL H_2O	24.75µL H_2O	5µL insert (long)
	3.0 µL 10x ligase	19.75µL H_2O
	1µL T4 DNA ligase (BRL 1U/µL)	3µL 10x
		1µL ligase

Electroporation of in vitro transcripts – BHK cells

5×10^6 cells / sample.

60-80% confluent BHK cells.

0.2 mm electrode cuvettes

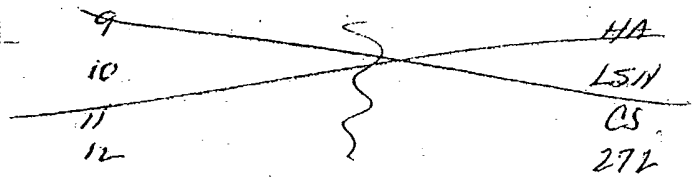
HA
LSN
CS
272-3

3-15-94

① Wash mono. layers with PBS + 2x
② 3ml trypsin
③ 10 ml MEM + 2% FBS
④ Pellet cells – 600 rpm, 5 min
⑤ Remove medium and resuspend cells in remaining fluid by tapping the tube.
⑥ Suspend cells in PBS
⑦ Repeat 5 & 6.

Using ~~Cuvettes~~ in 2 wells – depends on cell #'s.

Slide	#1	#2	Time	Exposure 15-LSN Control
1	Control	~~Control~~		
2	Control	~~Control~~	8 hrs	16
3	LSN ~~HA~~	~~HA~~ HA	8 hr	19, 18, 17 LSN sure
4	272 ~~CS~~	~~CS~~ CS		60" ~~15~~ 30
5	LSN LSN	HA	> 18 hrs	20, 21, 23 LSN 5 (), (), () 15" 30" 50"
6	272 272	CS ~~LSN~~		
7	LSN LSN	~~HA CS~~	> 28½ hrs	C3, 24 28 LSN 7
8	– 272-3	– CS 272		15 30 50



- Suspend cells at 1×10^7/ml in PBS
- 0.5ml / ~~cuvette~~ RNA
  10 µl RQA
- Pulsed by protocol 12:00 noon 3-15-94
- 1.5ml each into wells of 2-chamber Lab Tek's.
- Slides 1-4 washed w/ cold PBS 10 p.m.
- Day on bench overnight – Fix 2" in cold acetone
- Slides 5,6 harvested 8:00 a.m. Supernate from 272 transfection stored for later in replicon box Slot – 32
- Slides 7,8 Harvested 6:30 p.m. Supernate from 272 stored – 70 slot 33

3-16

3/13/94

From page 112

Transcription of pICS template:
DOA concentration 96ng/μl

5x	6 rxn
25mM NTP	9
^{3}H	2
H_2O	2
DOA	8
T7 Pol	3

31°C, 3 hrs.

1.5 hr samples.
$\frac{1}{3} = 11:30 - 2:30$

1 μl DOA = 10'

net
t_0 + 15107
t_0 + 16 hr
$t_{2\frac{1}{2}}$ + 2952 = 2790

18%

5 μg DOA

1094 cpm/ng

Phenol 2x
$CHCl_3$ 1x
NaOAc + EtOH
Resuspend in 50 μl H_2O
count 2 μl to determine [RDA] 480 240 cpm/μl
= 0.25 μg/μl

Synthesis of capped mRNA + helpers.

	Helpers		5x	RNTP (ACG) (25mM each)	CAP 20mM	GTP (2.5mM)	H_2O	^{3}H-UTP	DOA	Pol
①	Rsr I	200 g/ml	6	9	3	2.4	—	2	5	3
②	Rsr 2	74	6	9	3	2.4	—	2	5	
③	BstEII	391	6	9	3	2.4	2	2	3	
④	Spe I	148	6	9	3	2.4	0	2	5	
⑤	LSN	267	6	9	3	2.4	1	2	4	
					✓	✓	✓	✓		✓

1 μl samples
t_0 = 2:15 — 7:30

2mM GTP limiting
240a.4 cpm/μl

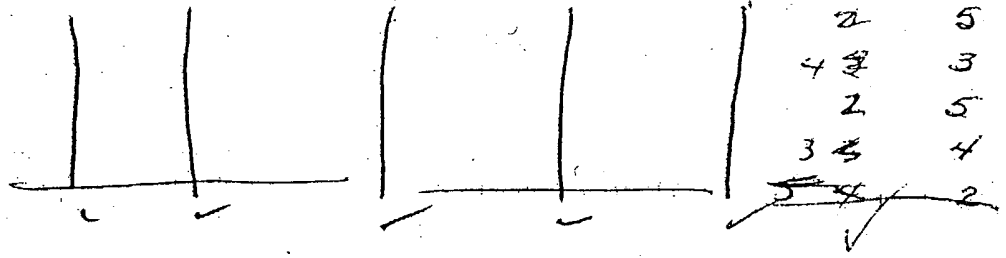
1)	36519	172	704	0.015
2)	28810	183	721	0.019
3)	37416	169	1815	0.044
4)	35478	220	1421	0.048
5)	40544	170	1118	.0233

Discarded
1.7 μg

Repeat — increase Cap to 0.5 mM
keeping GTP to same concentration 3mM

Repeat disease 476 - 0.3mM

	5x	NTP 25mM	CAP 20mM	25mM GTP	3H	H2O	DOA	P.C
1 Rsr 1	6	9	1	3	X	2	5	3
2 Rsr 2						2	5	
3 Bst+E2						4	3	
4 Spl 1						2	5	
5 LSN						3	4	
ZTZ							2	



1.5mL samples
37°C.
+ 9:00
+ 1:30 4.5hrs

total nMoles available
GTP limiting 300nM

	1	2	3	4	5
T	38165	22264	33462	39852	35026
BG	140	123	178	198	184
T-BG	1061	670	2257	2175	1702
Net	921	497	2079	1977	1518
%	0.024	0.02	0.062	.049	.0433
	7.1 ug	2.0	5.8	4.5	4.8

3 transfections Dot

Compartment #

#1	Rsr 1 .5uL	7.5μL LSN	5 spot 400μL cells in	
#2	Rsr 2 1.5uL	7.5uL LSN	each of tube chambers	
#3	Bst+E2 7uL	7.5μL LSN	(Vertical pairs	
#4	Spl 7uL	7.5uL LSN.	2nd slide of only controls	
			- Slide labeled C	
#5	LSN clone	7.5μL LSN	- 1.5uL to control slide	

HARVEST SUPERDATES FOR VIRUS TITRATIONS

3-31-94

Slide	Date	Time		
		1 2 3 4	7:10 a.m	3-31-94
1	4-1-94	8:00 a.m	+/+/+/+/+	VEE > 48hrs
2	4-1-94	6:30 p.m		
3	4-2-94	8:30 a.m	+/+/+/+/+	VEE > 72hrs
4	4-2-94	8:30 p.m	CPE w #1, possibly in others.	
C				
5	+/+/+/+/+	VEE Ag - Equivocal, very low efficiency,		
6				
7				
8				

LSN alone 4-1 8:00 Control

to page 127

fr pg. 116

Subcloning GPC Lasso

3/13/94 Ligations to Shuttle 2

II 1V:1i

1.25 Vector
10 µl insert (2ng)
14.75µl H_2O
3 µl 10x
1 µl ligase

III 1V:2i

1.25µl Vector
20 µl Insert
4.75µl H_2O
3µl 10x
1 µl ligase

RT; O/N.

No positives detected.
3-20-94
Alternative.
- Digest shuttle 2 with Hind3
- EtOH
- T4 repair
- BAP span column
- EtOH
- BAM HI digest — CIP Treat
- EtOH
- ~~Spun column~~
- Dilute to 30µl
OD 260/280 1.8/1.3 Store in Slot 61
OK by AGE

Incubate Hind3/Sca
Klenow repair to prove identity
30min if plasmid

42µl H_2O
5µl 10x3
3µl BamHI

LS1337
Apa I ~~Hind 3~~ digest — Apa I site is present in N-GPC intergenic sequence
Study doesn't Bam Linker added
- EtOH
- T4 repair
- BAP
- EtOH
- BAM Digest
- Prepare frag 1.5 Kb by electroelution - 2x EtOH
Store in Slot 100 -20°

See map of N done LS5560-7 3324/pg. 4

Ligation	Ins	Vector	10x	H_2O	Lig	
Frag 100	5	0	3	20	2	colonies
Vector (W)	0	2	3	25	2	
Vector+Frag	5	2	3	18	2	> Approximately equal #'s ~ 75
	✓	X	X	✓		

Prepare new vector preparations and phosphatase treat after each digestion.

~~4-5-91~~ 4-8-91

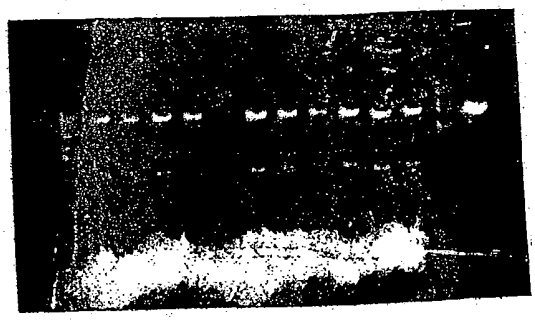
Rec'd ligation into JM105

Vector Control — 5 colonies

Vector + GPC — 12 colonies picked for mini's

Digest of BamHI EcoRI

#1 fragment GPC-Shuttle. — Slot 34



Remainder of #1 mini diluted to 50 ul and digested with ClaI

Fragment prepared by freeze/thaw

EtBr ppt

Ligate to vector

Sample	Insert	Vector	10x	H_2O	Lig
~~Vector~~ Vector	0	2	3	23	2
Vector + Frag	0	2	3	23	2

Fragment dried after EtBr ppt before sample used

4-9-91

One colony appeared

Digested with ClaI

fragment present

Cla fragment will contain RI site of MCS from shuttle

SmaI/RI will not cut

++ It appears that there is another RI site not seen previously ~~and both GPC frag. digested~~

Map by AsnI and KpnI digestion + Reption-Sea RI

KB

Rep-GPC ClaI SmaI/EcoRI

The second ~500 bp frag in the GPC Rep Sea/RI digest contains the fragment derived from the 3' end of the insert

T- 17a

Fact GPC is present as ClaI fragment

However VE structural gene is present as ~~2~~ 3 fragments

from page 114

5SP2 cloning cont'd.

Mun I digestion yields 1.6Kb frag from colonies D + J. —

What is this? Also present in Mun I digest of #4 on page 114.

Digest purified fragment with Mun I —

It's clear that the fragment is being partially deleted.

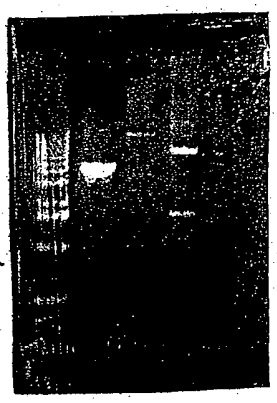
∴ Clone a smaller piece.

Continue analysis to determine if 5' end is present in D + J

① Digest with ClaI — again

②

— Discarded —



3-20-94 from page 74

Preparation of d1 helpers.

By direct site directed mutagenesis

① Prepare 5' 2000 bp Xba I – Sal I fragment from Helper Spl.
15 ug digested in 50 ul high salt.

② Electroelute Strain slot 95 - Diamond
③ Ethanol precipitate ~~Strain slot 100 = 20~~
④ Prepare Bluescript KS – and digest w/ Xba I + Eco R1
~~Control~~ No CIP Treatment
~~Pst~~
~~EtOH~~
~~OD260~~

Note – some undigested plasmid may be present in vector preparation

⑤ Ligate and transform 125's. plot 32
Has maroon phase 260/280 ratio

Ligation

	Insert	Vector	10x	H_2O	Lig	Do ligation the
Insert	5	0	3	20	2	flu dilution
Vector	0	3 ①	3	22-24	2	
KS + VE	5	3 ①	3	17 19	2	
	/	✓	4 ✓	4 x 17	4 ✓	

Rm T ~ 3:15

It appears that the vector Totox – Helper Spl co-runs with the 2000 bp insert. How will this manifest itself in the transformation? KD

The ~~Plasmid~~ control should indicate the problem. As the totox will have Xba R1 termini just as the fragment. May lead to a million colonies. Xba R1 Xba

Vector remnant can be cut with Bgl 1, Scal, Eam 1105 I etc.

15 ug helper Spl digested 0/N with Bgl 1 + Scal in high salt buffer XD Xbg R1

Purified by Geneclean #95 Diamond

3-21- As expected, many colonies appearing on plates screening on the purified fragment.

New vector prep. Repeat digestion 3-23-94

3-28 New approach

Cut the 2400 bp HindIII fragment out of
Adpw SpI
Ligate with pT2H and proceed w/ mutagenesis

Sample	Insert	Vector	NEB 10x	H2O	Ligase	# Colonies
FRAG	5	0	3	40	2	0
Vector #13	0	2	3	23	2	0
VE13-19	5	2	3	18	2	1 Blue, 25 white
	2 ✓	✓	4x3 ✓	4x18 ✓	✓	

- First 12 gave unrecognizable cuts w/ R1
- Pick additional 12 for H3 digestion while preparing up 19 Vector.

~~Preparation of~~ pT2H2R – deletion of T7 promoter

3/23/94 Made T7 delete oligo with correct sequence

Replica expression

300 ul removed from each well at time of collection (300µl from each paired well)

Vial #	Slide	Sample		
4-1-1	1	1	4-1-94	8:00 am.
4-1-2	1	2	Frozen at -70°C.	34
4-1-3	1	3		22hrs
4-1-4	1	4		
4-2-1	2	1		
4-2-2	2	2	4-1-94	32hrs
4-2-3	2	3		
4-2-4	2	4		

Treated 2 Echambers slides w/ 100µl virucells & away 60 hrs prior for particle formation

4-3-1	3	1		
4-3-2	3	2	4-2-94 8:10a.m.	46hrs
4-3-3	3	3		
4-3-4	3	4		
4-4-1	4	1		58
4-4-2	4	2	8:30pm 4-2 < plated 4-3-94	
4-4-3	4	3		
4-4-4	4	4		
4-5-1	5	1	Serum CPE in #1	
4-5-2	5	2	Serum CPE in #2	93hrs.
4-5-3	5	3		
4-5-4	5	4	4-3-94 11:30 a.m.	

4-3-94 Fixed plates for plaque assay

4-1-94 Two 8 well slides prepared for IFA of supernate

4-3-

Drink samples from 4-2-94 8:00am for IFA

Drink #20-6 and infect wells with 10-3, -4, -5 each 100µl of each. Slide A- #1, #2, Slide B #3 #4

#4-1-2 - 4-4 inoculated into 2 wells accordingly

4-6-1 | 6 | 1 | 8:30 a.m. 4-4-94
4-6-2 | | 2 | Explosive CPE in #1
4-6-3 | | 3 | Mural loss ~ 2-4
4-6-4 | | 4 |

4/4/94
- FA of Undiluted + 25% expansion ~ 5-10%
- Very low frequency of VEE Ag expression
  ±1%
  Is this transfection efficiency effect or lack of expression due to low frequency of viral infection

CONJ. # 288 0.1ml
MKV-anti LASSA
FOR USE ADD 1.9ml PBS
Dilution 1:20 9/89
USAMRIID

Conj #310 0.1Ml
Guinea pig anti VEE
For use add 1.5Ml PBS
Dil 1:16 2/91
USAMRIID/PAI

4/6/94
FA of diluted 48 hour samples indicate that particle titer ~ 4.5 logs for #1, (34) 2.
Repeat dilutions starting at undiluted
Controls are blank
Cells/well seeded

4/9
Dilute 36hr sample out and plate $10^{-4}, -3, -2, -1$

Slide A-1
#1 10^{-1} — 10^{-4} $\sqrt{2}$ 1.4×10^{3} 63×10^{3} 7×10^{4}
#2 " $< 10^{2}$ ~~63×10^{3}~~
3-1 #3 10^{-1} — 10^{-4} ~~69×10^{2}~~ $= 1$ 138×10^{2} 11×10^{3}
#4 10^{-1} — 10^{-4} 69×10^{2} $= 3$ 6.9×10^{3}

4/12/94 #4
Photos

6 pictures starting 26
Expo 4, 8, 15
1, 2 undil #1
3 10^{-1} #1

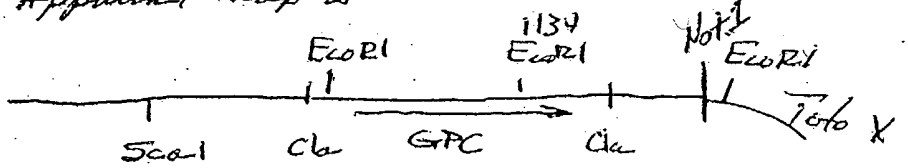
1 Page 13

4/9/94

GPC subcloning cont'd.

from 122

Apparent map is



To confirm, do single Asn I & Kpn I digests with respective controls

Start scale up.
Freeze culture in replicon box Shot 35 as GPC replicon

Asn I cut should release ~870 bp frag.
Kpn should release ~5.7Kb frag not seen in parallel replicon cut.

Kpn did not cut the replicon shown here - second cut showed that 1.5Kb frag is also present in the replicon
∴ Kpn cut is not useful

Kb	
Replicon	Asn I
GPC -	Asn I
Rep	Kpn I
GPC -	Kpn

Asn I cut - appears that there is another Asn I site not known about as all vector fragments are present as in control. And insert shows up as two fragments. Although two new fragments add up to more than expected for GPC - large 1.5Kb frag is as long as entire gene.
Still don't know orientation

1) Cut with BamHI -
2) Computer says Tofox has no PvuI sites

GPC 944
Replicon 1587, 2784 6300

Kb
Rep - PvuI
GPC - Rep PvuI
Rep BamHI/PvuI
GPC - Rep BamHI

Mapi = 925ng/ml 260/280 = 1.83
Cut 50ng DNA in Not I
50 additional mapping on page 134

Nov 29

Ind Extract of GPC w/ Lec

Sucrose Ind. Gravel Rod

[illegible] = 9.50 - 9.42 = .08/.10 = 18%

Slot 92

Transcription

1.5 c 5x 2 Flask

10 mM Tris 25 mM ATP - GTP

1 mM CaCl2

1 M KCl 25 mM GTP

2 M KCl 3 mM [illegible] 1.5 ml/2 ml/10

(87) 1.5 ml 2.0

2 ml ROA

3 ml [illegible] (Energy)

Precipitate

1.5 ml Sup 1.5 ml Sup

2 90 2 90

	1	9437			
	2	237			
xGPC	3	463			
	4	28746			
	5	155			
ROA	6	1180	1025	0.035	= 3 ug ROA - not much, repeat

6 pg - #33

Repeat transcription reaction

w/ intact Control

Why worse - cap reaction after so long?

	5x	25 mM GTP	3 mM ATP	25 mM GTP	3 M	1.5	ROA	Pl
Control	6	3	-	-	1			3
GPC -	6	3	-	-	1			3
GPC+	6	3	3	3	1			3

6 pg/ml = 183

4/15-97

Rif + h Sc Hc

from page 81

Vectn SL HB2 ... 60 25 mg/ml Rif ... Sal ... HB-Hb

Frag 97

79 Rif/Sal/Sc

Supplement	Frag	Vectn	HB2	H2O	Lig	Colonies
Vectn	0	1	3	24	2	250
Frag	5	0	3	20	2	5
Frag + Vectn	5	1	3	19	2	
	✓	✓	✓	×4		+ Sc Hc

Redo vectn

4/18/97

12 colonies picked + designated with Clark

#1, 5, 6, 8, 10, 12 release

fragment of appropriate size.

Hc 11/2 well with extremely runny growth.

Remainder of #12 digested with Clal for subcloning

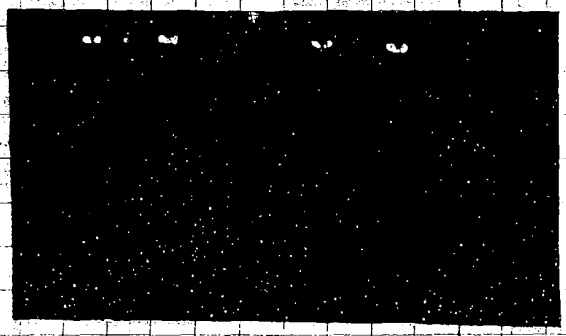
Frozen -70C in xpfhd box Via Rif Sc Hc 2

[Slot 36] Frag + slot 79

Final Check by restriction digestion

Hind3 digestion ✓ as expected - no photo

Clal



Apal ... R1 ... Sal ... R1 TH/HB T4 ... Not1

4/18

Ligation

	Vectn	Insert	HB2 10x	H2O	Lig
Vectn	2	0	3	23	2
Vectn + Rif	2	5	3	18	2
			✓	✓	

Sub DH5α 4-20-97 No colonies.

Repeat ligation 4-26-97

Frag	0	3	3	22	2
Vectn Rif	2 ✓	3 ✓	3 ✓	20 ✓	2 ✓

Remainder of frag stored in Slot 79

Sample	Insect	Vector	10x	40	Leg	
Deer	3	—	3	2	2	- contaminated w/ vector
Vector	—	2	3	13	2	- Pupae clean
Rift Rep	3	2	3	10	2	fragment.
	✓	✓	✓	✓	✓	

Ch. 1810 5x colonies picked away from 36411 page 11
5694 2 possibles - region Candroop Irrigated

from p. 124

In vitro transcription — GPC 4 preparations

Dot-blot hybridization of repeated transcription reactions

	10x	25mM NTP	25mM NTP	10x	25mM NTP	10x	DNA	Ambion Pel
Control	B	9	—	—	—	1	2	3
GPC-	B	9	—	—	—	1	5	3
GPC+	B	—	9	1	3	1	5	3

Control pT2 1B = 1 ug
GPC = 4 ug

Abbr — Ulang Ambion Buffer

	Ambion 10x	Ambion 25mM NTP	Ambion 10x	Ambion 25mM GTP	Pharmacia CAP	Pharmacia 3 NTP	DNA	Ambion Pel
Control	12	3	—	—	—	1	2	3
GPC-	12	3	—	—	—	1	5	3
GPC+		3	9	3	1	1	5	3

1.5 ul samples

✓ ✓ ✓ ✓ ✓ ✓ ✓

1:30

Samples at 30

Control	%	pMoles mc	ug		
12700					
763					
3825	0.18	16nM	53 ug		
GPC-					
12746					
256					
2313	0.1%	14.4 nM	41 ug ug	total remaining 395 ug/ml	
				0.5ml	
				= 1.6 ug/ml	
GPC+					
13160					
13968	too dark to read	12.6nM		Note that two samples	
1913	0.4%	4.15	4.15 ug	are very close in rpm.	

Seem previously

See page 130

Transfection
1) Day 2 expand
2) Freeze some Lin

2) Cells at 1/3
Harvest 48hrs
Count cells

Label helper transcription
1 3 4 1 - 2 - 1
3 - B + E2
4 - Spl1

Wash cells PBS
- 5ml trypsin - 5min
- Plate cells with
2ml GPC + 6ul 2 mg/ml 15ml medium
1ml helper + cap Wash and suspend
in 22ml PBS

Day 3 transfection
0.4ml into wells of one plate
3ml into each well of 6 well plate

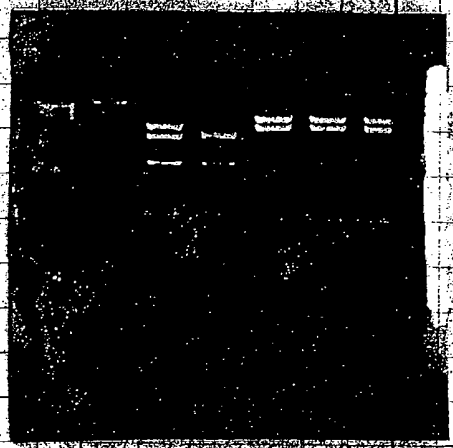
Well 1 - GPC alone
2 - GPC + helper 1 Rev1
3 - GPC + helper 3 B + E2
4 - GPC + helper 4 Spl1

Well 1 = GPC
2 = GPC + helper 1
3 = GPC + helper 3
4 = GPC + helper 4

Mapping

Not
1
R1

kb
GPC/p
Not1
Pvu2
Pvu2/Xh1
R1/Not
R1
Clu/R1



4-22-91 (Monday)

Cloning of helper Rsr2

Idea predicted that helper Rsr2 contains fusion of the NS2 gene with the Rsr2 gene. Repair by insertion of universal terminator.

Sat

① Cut 15ug helper 2 with Rsr2, Sac I
② Repair Sac I ends w/ p.f.

5µl 10x
2.5µl 5mM dNTP
40 5µl H2O
1µl T4
37°C, 20'

5µl EDTA, run on gel. Cut out bands. 4°C O/N.

Freeze-thaw

5-1

~~Ethanol ppt. digest with Not I~~

2/1 gel electroeluted, EtOH ppt. Resuspended both pellets in 100µl used 5µl for ligation w/ terminator universal.

Rsr II/universal terminator
5µl Rsr II
3µl u.t (20ng)
2µl 10x ligase (NEB)
10µl H2O
1µl ligase (NEB)

Rsr II alone
5µl Rsr II
12µl H2O
2µl 10x ligase
1µl ligase

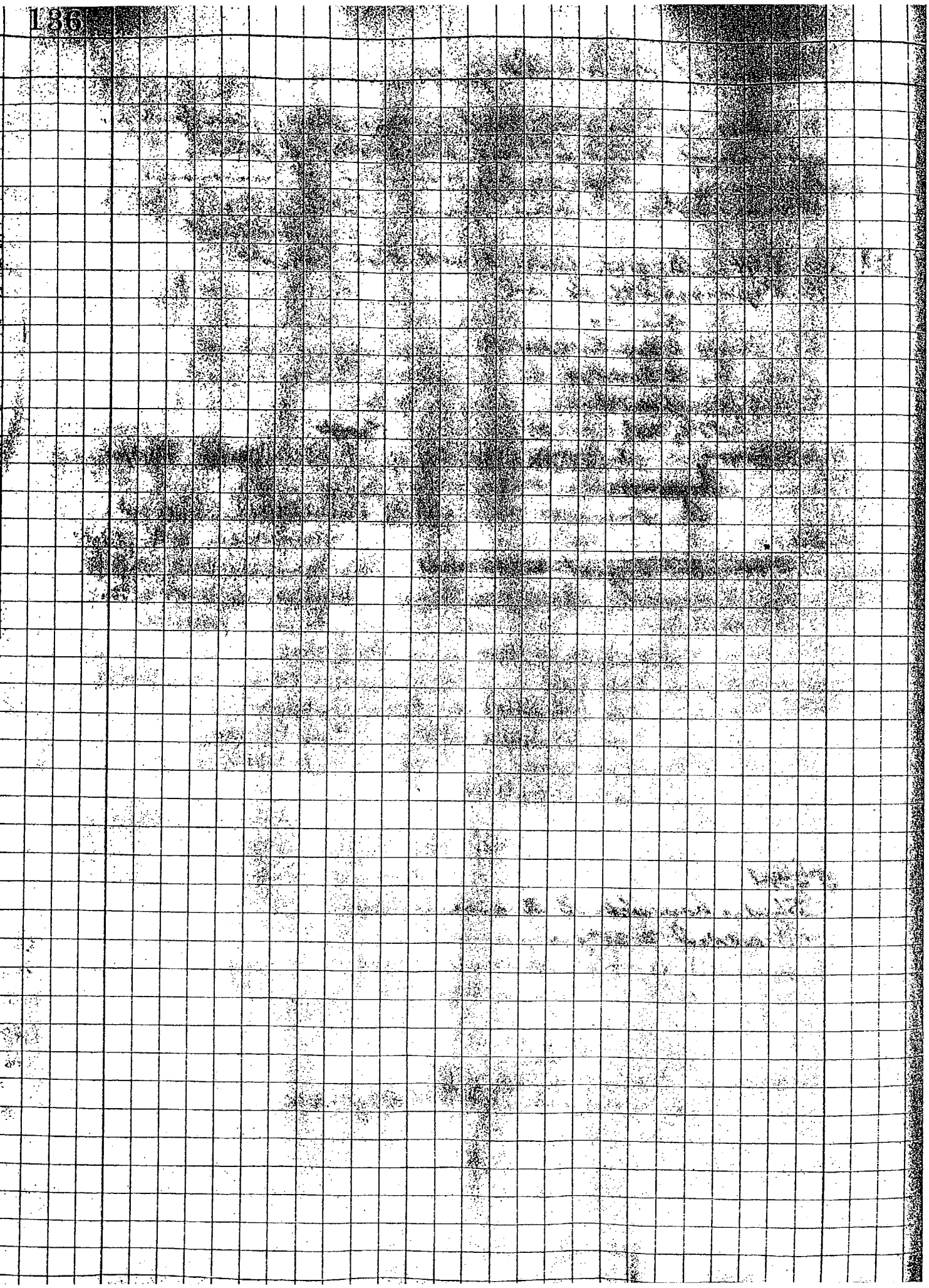
RT, O/N

5-3 gg

Single colony picked and digested with Asn I & Nsi I. Terminator predicted to contain Asn I site. Note decrease in size of 2.4 kb fragment.

Digest 3hrs at 37°C

Replica plate ~~and~~ Rsr2T
Mark standard ~~and~~ & store at 4°C.



4-28-94

Analysis of VOC in transfer pipettes.

Slides for analysis 2
Duplicates for same 2
Slides for QC samples 2
1 slide for control cells 1

7 slides, 28.
100 cells per well.

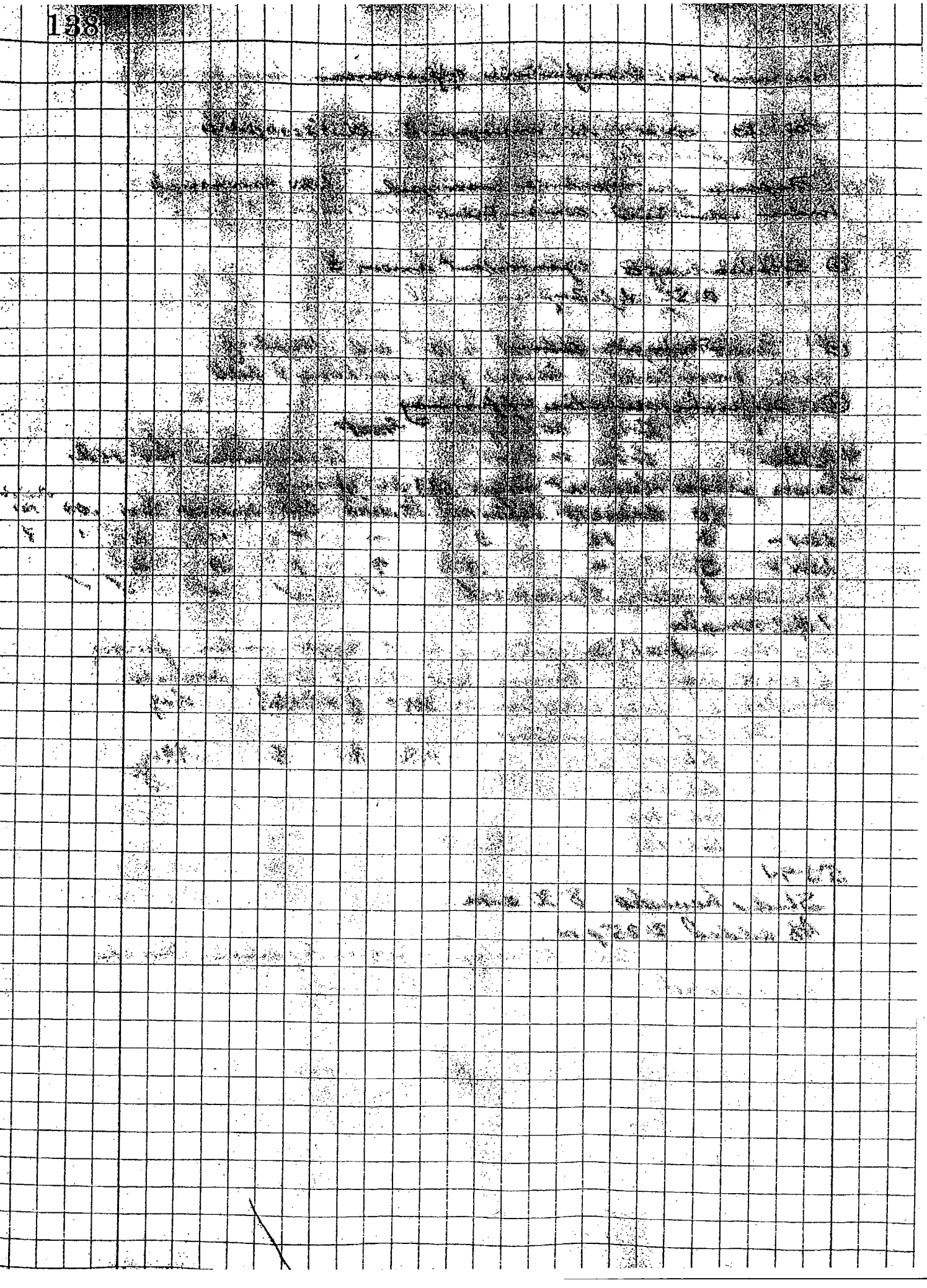
Small samples diluted to 10^{-3} and 100 µl of each available. Slides for one hour + add 100 µl fresh media.

~~6-1a~~ no help per Kent
6-1a + Ls-1 cells were too old
6-3a + B/82 for comp
6-4a + Dpl 1

~~Do same dilutions with~~
Repeat
Do the same dilutions with supernates from 6 well panels. Remember - Hoe was possible evidence for contamination of these.

Slides designated
6L-1a
6L-2a
6L-3a
6L-4a.

5-3-94
Slides harvested 8:30 a.m.
Ab added 2:25 p.m.
Cell monolayers were too dense. Should be repeated



Comparative transfection efficiencies

Plan to expand vs. unexpanded DNA to agarose

① Digest expand vs. unexpanded Lambda with Hind III cut Lambda

② Electroporate expanded + unexpanded

③ Grow out 24 hrs

④ Determine relative efficiency

Protocol Cam 6 on pg 139

	H2O	Hind III	Lambda	Kb	25mM Cap	Cap	25mM Cap	3 mM	DNA	And Xo
Lam +	6	12	3		—	+	—	2	4	3
Lam +	5	—	3		9	1	3	2	4	3
	1	—	1		1	1	1	1	1	

10 µl samples

6 = 12 xo

				Wt	%	n Mole	ng	
-cap	40781	1413	2505	109	5.2	46.8	15 ng	Dissolved
+cap	36774	503	2289	196	20	15	4.9 ng	Dissolved

Dilute each to 100 ng/µl

13.5 ug + cap remain in 27 µl ∴ add 108 µl H_2O

4.4 ug + cap remain ∴ dilute to 44 add 17 µl

Transfections

+cap 5 µl, 10 µl 0.5, 1 ug

-cap 5 µl 10 µl

- After electroporation, plate 2 wells of 8 well slide with each.
- Incubate 24 hrs, wash, fix & stain

Label slide 139 - page #

4 pg 43

5-3-99

→ - Cap increases efficiency greater than 50X

- With 0.5 ug plasmid + cells were too numerous to count + with 0.1 ug plasmid + cells were too numerous

4-30-94

9

Preparation of Rsr-1 for PREP-A cloning

① Digest 50 ug Helper Rsr1 in 100 ul high salt buffer with xba1 → Rsr1 5-4-94

② ~~Bacteria~~ Known titer 5ml overnight, 15 ml known 20', 37°C.

3) Phenol extract → Nat'l titer.

4) EtOH ppt. + store @ -20 20°C.

5) Electrophoretically purify and elute out material → Slot 113

6)

Digest 10 ug DNA with Xba1 EtOH, EtOH

⑦ 7½ Blunted - 1 minute 2% nuclease mix. 5ml 10x ✓
+5ml overnight 2.5ml enzyme ✓
37°C, 30' 41.5ml H2O ✓
5ml overnight 1ml 7½ ✓
Phenol
EtOH
Spun column
EtOH

⑧ Digest w/ Xba 37°C, 1hr. in 50ml high salt

⑨ Add 2.5ml 5 mM Tris pH 8.3
1ml BAP
37°C 30'

⑩ Phenol, EtOH

6) OD260/280 Slot 41 6 ug/ml

Ligation

Sample	Insert	Vector	VEB 10x	Kb	Ligase	Incubation
Insert	7	0	3	24	2	7500
Vector	—	5	3	8 20	2	16
Lig	7	5	3	19	2	7500
	/	✓	4X3	4X19		
5-4-94	X	X	X	X		

Lysate HB101

Fragment badly contaminated w/ vector

Repeat by same protocol but also digest with Bgl 1 with Xba.

5-6-94 Ligate as above.

5-7-94

- EtOH ppt'd.
- Suspended in 10 mM Tris
- Sal into Electroscomp Cells.

Twelve colonies picked.
digested with Bgl 2.

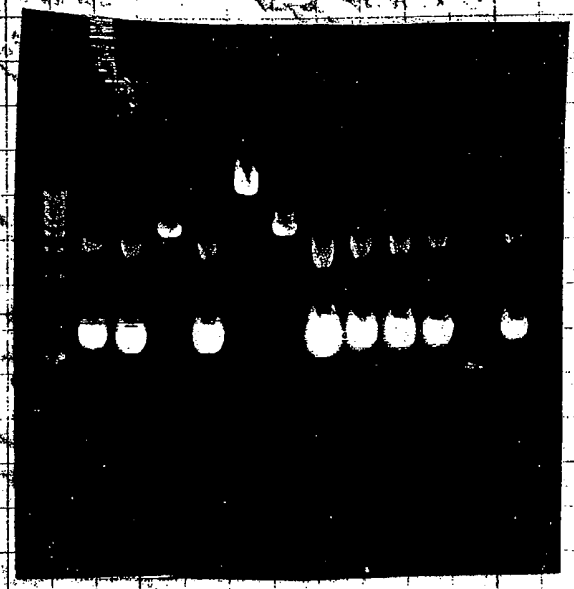
#'s 3, 4, 6 give expected products

∴ #3 fragments

pREP4 - Bam I

Shot 38 kpl on 1%

Start maxi prep



5-4-94

Scale up of LSN particle production from GPC

Results of last transfection indicate >90% transfected

∴ Repeat transfection with capt LSN and Helper Rsr

Split Buffs ~~5-4~~ 5-4-94

Transcription of LSN + Helper - Double Bun

Sample	H_2O	25mM HTP-GTP	Ambion 10x	25mM GTP	Cap	T-H	DOA	Ambion P.C.
LSN	12	18	6	6	2	2	8	6
Helper Rsr	10	18	6	6	2	2	10	6

37°C 4 hrs. 9:30 - 1:30

1.5ml samples

Should be repeated to make GPC capt also

GPC	17	18	6	6	2	2	3	6

2:30 - 6:30

Sample	CAM	Net	% n Mates	Inc ug	PDA	
LSN 1	31758					
2	185					~~Solubilized 0.5ug/ml~~
3	1479	1294	.041	12.4	4.1	3.5 14.6ul = 1ug
Helper						
1	20044					Frozen at -70°C.
2	205					
3	1591	1386	.046	14	4.5	3.8 13.4ul
						0.4ml
GPC						
1	33716					
2	268					Yields are low
3	1421	1653	.049	15	4.8	considering these are
					4.08	2x normal dry reactions
						12.5ul = 1ug

5-5-94

Electroporation - 2:00 p.m.

Slides 1 for GPC FA 1 VEE } 2 chamber 1.0ml each chamber

1 for LSN FA 1 VEE

- Seed the rest into one T-75 for particle production
- 1 well of 6 well panel for ^{35}S-labelling
- Remainder into T-75. - 7ml

See note pg 144

Note final time constant = 0.8.

Assays:
Plaque Assay -
FA - LSN GPC - serial dilutions
VEE

The remaining 7ml is not sufficient for a T75. Possibly enough for a T-25.

P.M. Split 1 T150 1:10 & 4 flasks for additional ~~flasks~~ starting with P5+25

Also seed 4 6well panels for plaque assay.
5-6 Seed 2-8 well slides for particle assay.
Collect supernates 5-7 a.m. Keep #8 as cell control.
Need to know if it's necessary to seed cells for FFA assay.
Process slides for FA starting at 11:30
Collect media from slides as
LSN 5-6-94 3516 144
GPC 5-6-94 3516 144

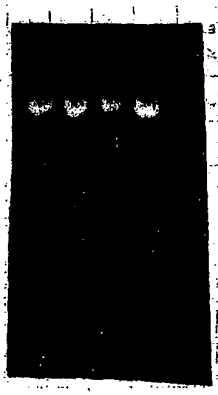
5-7-94 9:00 p.m.
Pronounced cytopathology in LSN culture much less in missing in GPC.
Harvest Fluids for Plaque Assays and FFA Assay.
Prepare two slides (8's) for FFA assay, 100K cells each well.

5-10
Titer of LSN particles $< 10^5$
PFU + may be PFU at $< 10^2$

GPC - transformation

5-15 Redo transcription of LSN
Need to increase efficiency of electroporation

to page 147



Cloning of Pst-1 + BstEz 5' for cloning US ORF.

For BstEz prepare 5' XbaI - SacI fragment - 1.6 KB fragment
Pst-1 5' XbaI - BstEz fragment - 2.2 frag
Needle XbaI - Pst upto 30 M

Shred gel slab 123 respectively.

Into pIDZ - 1-13-94 4.15 p.m.

Ligation

Sample	Vector	Insert	10x	4/0	Ligase	
pTdt2 X/P	2	—	3	25	2	13 wkte
pTdt2 X/S	2		3	25	2	~100
Rsr X/P	2	5	3	18	2	~100
Bst X/S	2	5	3	18	2	~100

- 2° fragment contaminated with vector. Can be cleaned up with 2nd digestion

- Still having 2° fragment problems. Will attempt to provide the needed fragment for both Rsr + Spl ligations.

Redo Xba/Sal digestion of vector

Xba + Sal sites are too close together

Do multiple ligations in pBluescript

Using KS-, primer can be positive sense. Forward primer = T7 primer

Digest Lig KS- with Xba + Sal and redo ligation

5-17

10-15 a.m.

Sample	Insert	Vector	10x	H2O	Lig	
Trig	5	—	3	20	2	0 colonies
Vilm	—	2	3	23	2	~100 colonies / white
Trig	5	2	3	18	2	~150 colonies / many white

Pick 12 Trig digestion

All are positive by Xba/Sal digestion

Inoculate #2, 3 into 2 ml 2xYT for miniprep

Inoculate #2 in replicate into 5 ml 2xYT

5-21

CIP'd w/ #2 blasted for Q

Trig digest in slot 40 ng/lane Lig #2 discarded 6-2-94

Sequencing DNA in slot 109

5-24 Purchase SDM

6 pmole 149

3-18-94

Transcription from 3-9

	H₂O	Ambion NTP	Ambion 10x	25mM NTP	CAP	25mM GTP	3H	DOA	Amber DE
L34-	1/8	1/2	3	—	—	—	2	2	3
25NT	7	—	3	9	1	3	2	2	3
	DOA = 1 ug/ml		✓	✓	✓	✓			1 ug

37°C. 4 @ 11:30 - 4:45 1 ml samples
Trypsin 10x of the last sample

	to	bf	tx	rxt	%	NTPs	Ug	DOA	ug	RNA/ml
-	3.15x	25	1.9x		0.15	1.25		2		1.4
+	3.5x	28	1.8x		0.25	1	2.2ug			0.16

About the normal yield

3-16-94
Transcription
Two T-150's
after 1st 785 wash complete media was added immediately & then 2x more.
Transfect by usual protocol.
Harvest,
cells combined with PDA and immediately electroporated. (PDA and human cells added to PDA in cold tube and immediately transferred to cuvette.

Two cells mixed w/ 0.4 ml/each
2:30 pm

0.25	0.50	1.0
Blank		

0.25 = 2.5 ul
0.5 = 5 ul
1 ug = 10 ul.

3-17
1st time at 9:15 a.m.
only the 0.25ug sample gave clear staining.
~10 % cells were staining.
The 0.5 & 1.0 cells were stained at just as fluorescence.

6-25-94

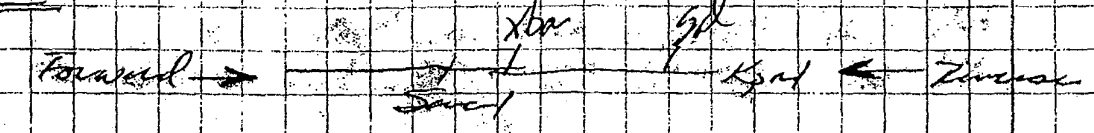
Mutagenesis of Kbp 225 ATG. from page 146

6 colonies picked

5 sequenced

- Mini done by Wizard Rep Kit
- DOT denatured
- Annealed Kinase primer cocktail & sequenced.

Remember chart is in the 5th tube of KS-



All turned out to be CHE2 clone.

Is the digesting DOA the proper VEE clone or is it CH.

∴ Do PCR to see if it can be amplified with CHK or VEE primers.

VEE primers.

Helper 2	8-39	D-3	32	49 μM
Helper Reverse	353-331	A-3	24	94 μM

CHK primers

CHE2 Gap pairing

CHK gap 1	U	20 μM	26	
CHK gap 2	T	69 μM	26	

PCR		A	B	C	D	
H_2O		15.3	13.3	11.9	69.9	✓
10X	10					✓
dNTP	5					✓
Primers						
Helper 2		2	2			✓
Helper Rev		1.1	1.1			✓
CHK gap 1				5	5	✓
CHK gap 2				1.6	1.6	✓ 5
Mg Cl		4	8	4	8	
Taq	0.5					
DNA	2					

Program 55

Repeat

Do autopsies 39-40 both have CHK tasks

Plan VE 5' x/5.

Amplify as on previous page

Substrate: 2nd minipep results 1%

A,B = 39 = E,F AC - 1mM Kg

CD = 40 = G,H BD = 1mM Kg

A-D = 1mM Kg

E-H = 1mM Kg

		39		40		
		A	B	C	D	E F G H
H_2O		113	693	665	665	✓
10×	10					✓
enzyme	4					✓
Primers						
Helper 2		2	2	2	2	✓
Hoffmeister		1.1	1.1	1.1	1.1	✓
CHK26-1		~~5~~	5	5	~~2~~ 5	✓
GHK26-2		1.1	2.1	5	5	1.1 ~~5~~ 5	✓
Tag	0.5					
MgCl2		4	8	4	8	
DDA	5	H2O				

Positive bands in lanes 2-7 only.

Suggests that both are CHK

Go back & cut 39 mini pep with

Sty 1 - cuts CHK

Pst 2 - cuts VEE.

Redo transformation of CJ236 and repeat mutagenesis - new colonies frozen in 86 of 40

Mutagenesis repeated 6-2-94 and into JM105

6-6

Four colonies picked

Digested w/ X=5, only 1 of 4 release

Paper fragment. ! Sequence 6.2.4 with forward.

Two of the cleared preps contained large amount of resin after OH⁻ + EtOH. Wires HD would. Proceed with sequencing with forward primer and lab mixed reaction buffer.
No readable results.
Repeat prep + OH⁻ denaturation and redoing 6-8-84.
All were wild type

6-9- found that oligo was wrong polarity
Prepare nucleic complement and repeat mutagenesis.
6-8-84 Left JH 10:15 a.m.

6/10/84

152

Primer map 1st = 1 map

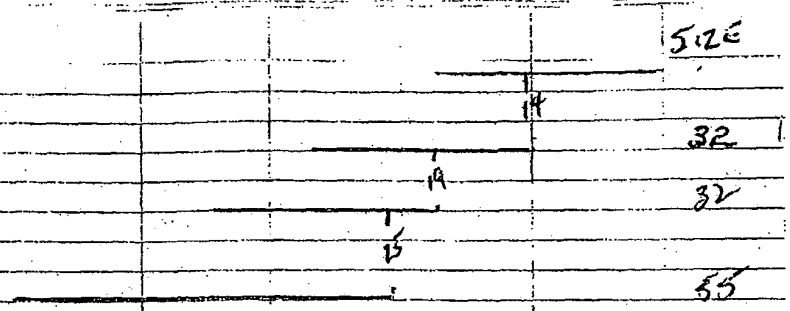
#1 Page 22

Helper 1

Helper 2A, 2B

Helper 2B

Helper 3



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